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Comparison of Age-Adjusted Cancer Incidence Rates in Denmark and the United States [1, 2]

JOHANNES CLEMMESEN, *M. D., and* ARNE NIELSEN, *Lic. act.*, Cancerregisteret,[3] Copenhagen, Denmark

It appears from studies during the last decade that differences in incidence of malignant diseases are difficult to demonstrate with absolute certainty within a single country, so that in most cases it will be necessary to study such differences, and subsequently their causes, through international collaboration by the two or more agencies directly responsible for collection of the data because only the agencies involved will be able to comment fully upon sources of error that inevitably enter into such comparisons.

However, for preliminary comparisons preceding more detailed study, it will be useful to examine figures adjusted for differences in age distribution, since this factor can have an important effect on the crude incidence rates for malignant disease. For this reason we have age-adjusted our incidence figures for Denmark, for the periods 1943–47 and 1948–52, on the basis of a standard population (the United States census population of 1950) used in the presentation of recent data from the United States (*1*).

The tecbnique employed in the collection of the material has been published in detail elsewhere (*2*) and so have the figures on the age distribution of the Danish population at risk (*3*) for the period concerned. Earlier publications on cancer incidence in Denmark for 1942–44 have been published by Clemmesen and Busk (*4–6*) and for 1943–47 by Clemmesen and Nielsen (*7*). Incidence figures on cancer of the lung were published by Clemmesen, Nielsen, and Jensen (*8*) and on cancer of urinary organs by Clemmesen and Nielsen for 1943–53 (*9*), who have also presented data on the number of cases reported for various habitation areas in Denmark for the same period (*10, 11*). Further figures for the period 1943 to 1953 will be published in *Danish Medical Bulletin* and elsewhere. Figures for the latter period have been based on renewed revision of the basic material, so far as 1943 to 1947 is concerned.

In the present paper age-adjusted rates have been computed for each sex separately for the Capital, Capital Suburbs, Provincial Towns, and

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[3] Strandboulevarden 49, København Ø, Danmark.

Rural Areas for two 5-year periods, 1943–47 and 1948–52 (tables 1 and 2) on the basis of the United States standard population for 1950.

If we consider the total for malignant neoplasms we find the highest incidence in the Capital and the lowest in Rural Areas, while Provincial Towns and the less important category Copenhagen Suburbs take an intermediate position. Although the total figures for malignant neoplasms may be of practical value, we find at the moment no evidence justifying such summation of diseases of varying etiology, symptoms, treatment, and prognosis, for the purpose of endemiological studies. Thus, although we find an increase for men for neoplasms of all sites from 1943–47 to 1948–52, which is most pronounced for the Capital and less for Provincial Towns and Rural Areas, and a decrease for Copenhagen Suburbs, an explanation of this tendency must rest on an analysis of the individual sites. Similar views apply to the slight decrease for women in all areas except the Capital where a small increase is suggested.

The individual neoplastic diseases display, as a rule, a tendency to higher rates for the Capital and lower for Provincial Towns and Rural Areas, although there are some exceptions:

1) *Cancer of the lip* shows the highest incidence in Rural Areas and lowest in the Capital for men, which is consistent with the theory that the effect of sunlight enters as an etiological factor. Among men this disease has increased in the Capital and decreased in Provincial Towns.

2) *Skin cancer* incidence among men and women has similarly risen for the Capital, which now shows a slightly higher incidence than Rural Areas.

3) *Gastric cancer* shows higher incidence for Rural Areas than for the Capital for both sexes—a tendency opposite to findings in England and not without exception since the values for Provincial Towns are highest. Recently, Grandjean has established that the slight downward trend for this neoplasm on the whole applies only to cases not examined in hospitals, so that it may be assumed that the efficiency of diagnosis may influence figures for this disease (*12*).

4) *Bronchial carcinoma* has been increasing among men in Copenhagen since 1930 and in Provincial Towns and Rural Areas since about 1938 and 1940 (*8*), and it is evident that incidence is highest in the Capital followed by Provincial Towns and Rural Areas.

Analysis of incidence rates for women at a time when no substantial rise had appeared for this sex, indicated, however, that some part of the higher incidence for this sex in the Capital might have been due to better diagnosis or other influence. The differences for women were small compared to those noted for men (*loc. cit.*). It should be noted, however, that a rise now has appeared for women in Copenhagen (*13*).

5) *Mammary cancer* conforms to the pattern observed elsewhere, being most frequent in the Capital where fertility is lower than in the rest of the country (*14*). It is true the findings hold for both sexes, but the number of cases reported for men are too few to allow conclusions. The same observation applies to the change in rates.

6) *Cervical cancer*, however, as previously demonstrated by Clemmesen in 1951 (*14*), and contrary to experience elsewhere, shows the same tendency although on a decreasing scale. This phenomenon has been ascribed to artificial abortions. If we assume that a promoting influence of sexual activity on cervical carcinoma takes place before pregnancy, and conversely suggest a preventive effect from lactation on mammary cancer, a high number of abortions will tend to give the population "the worst of both" (*15*).

7) *Multiple myeloma* and *lymphatic leukemia* for both sexes tend to show slightly higher figures for the Capital.

Changes in incidence during the two periods 1943–47 and 1948–52 are most pronounced for the following categories:

a) Cancer of the mouth among men has decreased, except in Rural Areas.

b) Cancer of the esophagus has decreased for both sexes.

c) The increase observed for the smaller categories of liver and pancreas may be taken with a grain of salt.

d) The increase for bladder tumors among men in Copenhagen has caused a special investigation (Clemmesen and Nielsen 1956, *9*).

e) The increase of prostatic carcinoma was discussed at some length in the paper quoted (*9*) and cannot at the moment be unreservedly accepted as reflecting a true increase in risk, because diagnostic factors may have considerable influence on the number of cases reported.

Discussion

Since the figures for Copenhagen offer the best possibilities for comparison, we have limited ourselves to a discussion of the Copenhagen cancer incidence and the corresponding rates for the 10 American cities studied by the National Cancer Institute (*1*) that appear in the tables.

It should be noted that it is difficult to assess the significance to be attached to differences in rates. Such assessment will as a rule require detailed study of the materials on which figures are based, and it is the aim of the present paper to draw attention to the possibilities for such scrutiny.

Buccal Cavity and Pharynx

Both sexes show higher rates for whites and nonwhites in cities of the United States than in Copenhagen. This tendency appears to affect all sites of tumors in this group, with the exception of lip for which nonwhites show the lowest incidence. A similar but more questionable trend appears for the small category of oral cancer.

Digestive Organs and Peritoneum

White men show approximately the same incidence in both countries, with somewhat lower rates appearing for nonwhite men, while white women in the United States show slightly higher and nonwhite women slightly lower incidence than found in Copenhagen.

TABLE 1.—*Age-adjusted* cancer incidence rates per 100,000 male population by primary site: urban and rural areas of Denmark, 1943–47 and 1948–52, and 10 metropolitan areas of the United States, 1947†*

W. H. O. code no.‡	Site	Denmark								U. S. A., 10 cities	
		Capital		Provincial towns		Rural areas		Copenhagen suburbs		White 1947†	Non-white 1947†
		1943–47	1948–52	1943–47	1948–52	1943–47	1948–52	1943–47	1948–52		
140–145, 147–148	*Buccal cavity and pharynx*	*9.6*	*8.4*	*9.0*	*7.6*	*9.5*	*10.1*	*11.2*	*6.8*	*21.1*	*10.9*
140	Lip	1.8	2.9	5.9	4.7	7.8	8.1	4.8	3.8	5.8	0.4
141	Tongue	1.5	1.4	0.6	1.0	0.2	0.3	1.4	0.9	4.2	2.7
142	Salivary glands	0.3	0.9	0.8	0.3	0.6	0.8	—	0.0	2.1	2.5
143, 144	Mouth	2.0	1.3	1.0	0.8	0.6	0.6	2.1	1.2	4.6	2.0
145, 147	Tonsil (throat), pharynx, hypopharynx and unspecified.	4.0	1.9	0.7	0.8	0.3	0.3	2.9	0.9	4.4	3.3
148 U. S. incl. 146											
150–159	*Digestive organs and peritoneum*	*117.0*	*110.3*	*108.9*	*100.2*	*91.0*	*84.1*	*124.9*	*94.4*	*110.5*	*96.9*
150	Esophagus	10.5	8.0	6.4	5.1	5.0	3.8	7.2	9.2	8.3	9.7
151	Stomach	41.7	37.0	50.5	43.4	46.1	41.8	58.9	35.0	34.1	39.6
152	Small intestine	1.1	0.5	0.6	0.6	0.3	0.3	0.7	0.5	1.0	0.7
153	Large intestine	22.9	22.8	19.0	19.3	14.3	14.0	23.6	21.1	26.2	13.9
154	Rectum	26.9	25.2	23.3	22.5	18.3	17.4	24.5	17.0	22.1	12.7
155.0	Liver	5.0	5.4	4.0	3.4	2.6	2.0	1.9	3.4	4.9	5.3
155.1	Biliary passages	2.6	2.8	0.6	1.0	0.4	0.6	1.4	1.8	2.3	1.0
157	Pancreas	5.5	7.7	3.8	4.2	3.6	3.6	6.2	6.0	9.3	11.6
158, 159	Other and unspecified digestive organs.	0.8	0.9	0.7	0.7	0.4	0.6	0.5	0.4	2.3	2.4
160–164, 146	*Respiratory system*	*36.0*	*50.7*	*14.5*	*20.0*	*7.8*	*10.6*	*25.6*	*36.4*	*39.0*	*33.8*
160, 164	Nasal cavities, mediastinum, maxillary and jawbone	1.9	2.2	1.3	1.4	1.1	0.8	2.7	1.2	1.8	2.5
161, 162.0	Larynx and trachea	2.9	3.6	1.8	2.2	1.0	1.0	2.8	3.5	7.7	5.9
162.1, 162.2	Lung	30.6	43.6	10.5	15.3	5.0	8.3	18.7	30.4	29.5	25.4
(163 or 231)	"Tumor" of lung	0.6	1.3	0.9	1.1	0.7	0.5	1.4	1.3	—	—
170	*Breast*	*0.5*	*0.6*	*0.4*	*0.6*	*0.3*	*0.3*	*0.2*	*0.5*	*0.8*	*0.2*

177–179	*Male genital organs*	*22. 5*	*27. 1*	*18. 8*	*22. 0*	*15. 7*	*19. 3*	*16. 1*	*18. 5*	*38. 6*	*54. 0*
177	Prostate	17. 3	22. 2	13. 1	17. 0	11. 9	15. 0	13. 6	14. 9	34. 8	49. 9
178, 179	Other and unspecified	5. 2	4. 9	5. 7	5. 0	3. 8	4. 3	2. 5	3. 6	3. 8	4. 1
180–181	*Urinary organs*	*17. 5*	*24. 3*	*11. 3*	*12. 1*	*7. 7*	*9. 3*	*13. 0*	*18. 1*	*24. 5*	*11. 5*
180	Kidney	7. 8	8. 8	4. 4	4. 4	3. 2	3. 7	4. 1	5. 3	5. 6	4. 3
181.0 Denmark incl. papilloma.	Bladder, carcinoma and papilloma.	9. 7	15. 3	6. 9	7. 7	4. 5	5. 6	8. 7	12. 4	18. 6	7. 0
181.8	Ureter and urethra	—	0. 2	0. 0	0. 0	0. 0	0. 0	0. 2	0. 4	0. 3	0. 2
190–191	*Skin (incl. melanoma)*	*16. 9*	*24. 0*	*21. 6*	*26. 7*	*19. 8*	*23. 2*	*23. 1*	*19. 9*	*56. 1*	*4. 5*
193	*Brain and nervous system*	*8. 2*	*7. 6*	*5. 1*	*5. 8*	*4. 8*	*5. 3*	*6. 2*	*4. 8*	*7. 1*	*4. 4*
193.0	Brain	6. 6	6. 5	4. 4	5. 1	4. 5	4. 8	5. 2	3. 9	5. 9	3. 1
193.1–193.9	Other parts	1. 6	1. 1	0. 7	0. 7	0. 3	0. 5	1. 0	0. 9	1. 2	1. 3
192, 194–196	*Other sites*	*3. 8*	*3. 7*	*2. 6*	*2. 2*	*2. 1*	*1. 5*	*0. 7*	*1. 4*	*5. 2*	*3. 2*
192	Eye	1. 4	1. 0	0. 9	1. 0	1. 0	0. 7	0. 3	0. 4	1. 3	0. 2
194	Thyroid	0. 8	1. 2	0. 3	0. 5	0. 3	0. 3	—	—	1. 2	0. 2
195	Suprarenal gland, thymus	0. 3	0. 5	0. 2	0. 1	0. 0	0. 0	—	—	0. 8	0. 8
196	Bones and joints (sarcomas)	1. 3	1. 0	1. 2	0. 6	0. 8	0. 5	0. 4	1. 0	1. 9	2. 0
199	*Unspecified sites*	*3. 2*	*2. 6*	*4. 3*	*3. 7*	*3. 7*	*3. 2*	*3. 2*	*3. 5*	*13. 4*	*12. 7*
156 partly 165, 198, 199. 6	Metastasis	4. 0	4. 4	4. 0	3. 2	3. 2	2. 7	3. 6	2. 5	—	—
197	Connective tissue	1. 4	2. 5	2. 1	2. 7	1. 5	1. 9	1. 6	1. 6	2. 1	2. 6
204	Leukemia	7. 2	8. 4	7. 8	7. 0	5. 7	6. 6	5. 9	5. 2	9. 1	8. 6
201	Lymphogranulomatosis, Hodgkin	2. 4	2. 2	1. 7	2. 6	2. 2	2. 5	1. 2	1. 8	3. 4	2. 7
200, 202, 203, 205	Denmark incl.: All sarcomas of various sites except bones and joints.	6. 3	8. 0	5. 9	8. 3	4. 8	5. 1	5. 2	5. 8	7. 4	6. 6
	All malignant neoplasms	256. 5	284. 8	218. 0	224. 7	179. 8	185. 7	241. 7	221. 2	338. 3	252. 6

*For both Danish and American data the direct method of age-adjustment was employed. The standard age distribution used was that of the total population of the continental United States in 1950 (*1*).

†Data for 6 areas were collected in 1947; data for the other 4 areas were collected in 1948 (*1*).

‡In the Danish material sarcomas diagnosed as such have been excluded from tumors assigned to the individual sites, except for bones and joints, 196. This applies also to lymphosarcomas and has been done in order to make age-distribution curves for carcinomas as accurate as possible. U. S. figures include all malignant tumors for each site. It is estimated that the differences between the 2 countries caused by this deviation in procedure will be insignificant to the present study.

TABLE 2.—*Age-adjusted* cancer incidence rates per 100,000 female population by primary site: urban and rural areas of Denmark, 1943–47 and 1948–52, and 10 metropolitan areas of the United States, 1947†*

W. H. O. code no.‡	Site	Denmark								U. S. A., 10 cities	
		Capital		Provincial towns		Rural areas		Copenhagen suburbs		White 1947†	Non-white 1947†
		1943–47	1948–52	1943–47	1948–52	1943–47	1948–52	1943–47	1948–52		
140–145, 147–148	*Buccal cavity and pharynx*	*3.5*	*3.7*	*2.3*	*2.2*	*2.6*	*2.0*	*2.0*	*3.3*	*6.3*	*5.3*
140	Lip	0.4	0.3	0.3	0.2	0.6	0.3	0.3	0.5	0.8	0.2
141	Tongue	0.9	0.6	0.8	0.5	0.4	0.5	1.3	0.8	1.2	1.1
142	Salivary gland	0.7	1.6	0.6	0.6	0.5	0.5	0.4	0.8	2.0	2.6
143, 144	Mouth	0.8	0.8	0.3	0.5	0.5	0.4	—	1.2	1.5	0.5
145, 147, 148 U. S. incl. 146.	Tonsil (throat), pharynx, hypopharynx, and unspecified.	0.7	0.4	0.3	0.4	0.6	0.3	—	—	0.8	0.9
150–159	*Digestive organs and peritoneum*	*74.9*	*72.8*	*81.7*	*70.7*	*80.3*	*69.4*	*72.7*	*70.3*	*79.8*	*68.0*
150	Esophagus	2.6	1.6	2.9	2.4	3.2	2.1	4.7	2.0	1.9	2.2
151	Stomach	25.9	23.6	35.2	26.7	38.0	29.8	25.1	24.0	18.3	22.8
152	Small intestine	0.7	0.4	0.5	0.4	0.5	0.4	0.4	0.3	0.6	0.2
153	Large intestine	18.1	21.0	19.1	17.8	17.2	16.4	16.8	22.7	27.8	16.2
154	Rectum	15.1	13.9	11.9	12.2	11.7	11.2	12.1	10.2	15.2	13.6
155.0, 156 partly	Liver	4.0	3.0	5.4	3.0	4.4	3.4	4.8	1.8	4.2	2.7
155.1	Biliary passages	3.2	3.6	2.2	2.7	1.5	1.8	3.7	1.7	4.4	2.0
157	Pancreas	3.5	4.6	3.3	4.2	2.9	3.1	4.1	5.1	5.5	6.4
158, 159	Other and unspecified digestive organs.	0.8	1.1	1.2	1.3	0.9	1.2	1.0	2.5	1.9	1.9
160–164, 146	*Respiratory system*	*6.4*	*7.2*	*4.2*	*4.4*	*4.1*	*3.9*	*5.2*	*9.0*	*8.4*	*7.9*
160, 164	Nasal cavities, mediastinum, maxillary and jawbone	0.9	0.6	1.1	0.8	0.5	0.6	0.3	1.5	1.3	1.5
161, 162.0	Larynx and trachea	0.5	0.3	0.3	0.1	0.4	0.2	—	0.5	0.6	0.6
162.1, 162.2, 163	Lung	4.2	6.1	2.4	3.3	2.6	2.7	4.4	6.2	6.5	5.8
(163 or 231)	"Tumor" of lung	0.8	0.2	0.4	0.2	0.6	0.4	0.5	0.8	—	—
170	*Breast*	*56.9*	*57.4*	*51.4*	*49.3*	*38.8*	*40.9*	*56.4*	*52.4*	*72.6*	*53.9*
171–174	*Uterus*	*51.1*	*53.9*	*46.0*	*47.4*	*26.9*	*29.5*	*50.9*	*43.9*	*56.3*	*95.6*
171	Cervix	38.3	38.4	32.9	34.9	17.2	20.2	38.3	30.3	32.8	70.4

172	Corpus	10.7	14.4	10.3	10.9	7.2	7.9	10.3	12.3	10.3	11.2
173, 174	Other and unspecified	2.1	1.1	2.8	1.6	2.5	1.4	2.3	1.3	13.2	14.0
175–176	*Other female genital organs*	*17.0*	*18.0*	*15.2*	*15.2*	*11.0*	*13.2*	*13.5*	*14.4*	*18.0*	*12.8*
175.0	Ovary	14.1	15.3	11.9	12.3	9.0	10.8	11.9	10.6	14.7	9.9
176.0, 176.1	Vulva, vagina	2.6	2.3	3.3	2.7	2.0	2.2	1.6	3.7	3.0	2.9
175.1, 176.8, 176.9	Other and unspecified	0.3	0.4	0.0	0.2	0.0	0.2	—	0.1	0.3	—
180–181	*Urinary organs*	*8.0*	*9.4*	*5.5*	*5.6*	*4.9*	*5.5*	*5.7*	*6.5*	*11.5*	*11.7*
180	Kidney	4.3	5.2	2.9	3.0	2.9	3.4	1.6	2.8	3.1	3.0
181.0 Denmark incl. papilloma.	Bladder, carcinoma, and papilloma.	3.4	4.1	2.5	2.4	2.0	2.1	3.9	3.7	8.0	7.9
181.8	Ureter and urethra	0.3	0.1	0.1	0.2	—	0.0	0.2	—	0.4	0.8
190–191	*Skin (incl. melanoma)*	*13.3*	*16.9*	*15.0*	*16.5*	*13.1*	*14.4*	*11.7*	*14.1*	*39.5*	*6.0*
193	*Brain and nervous system*	*6.9*	*6.6*	*4.4*	*3.9*	*4.5*	*4.5*	*5.6*	*6.0*	*5.0*	*3.6*
193.0	Brain	4.8	4.6	3.3	3.1	3.7	3.5	4.4	3.9	3.8	2.7
193.1–193.9	Other parts	2.1	2.0	1.1	0.8	0.8	1.0	1.2	2.1	1.2	0.9
192, 194–196	*Other sites*	*3.0*	*2.8*	*2.3*	*2.6*	*2.2*	*2.5*	*2.7*	*1.5*	*6.6*	*5.5*
192	Eye	0.8	0.8	0.6	0.7	0.6	0.7	0.4	0.4	1.0	0.4
194	Thyroid	1.3	1.4	1.1	1.1	0.9	1.1	1.7	0.4	3.4	3.3
195	Suprarenal gland, thymus	0.2	0.3	0.0	0.2	0.0	0.1	—	0.1	0.4	0.4
196	Bones and joints (sarcomas)	0.7	0.3	0.6	0.6	0.7	0.6	0.6	0.6	1.8	1.4
199	*Unspecified sites*	*7.1*	*4.6*	*8.6*	*7.1*	*9.1*	*6.9*	*8.4*	*7.9*	*13.8*	*11.5*
156 partly, 165, 198, 199.6.	Metastasis	2.9	2.6	3.8	2.5	3.0	3.0	1.9	2.3	—	—
197	Connective tissue	0.8	1.7	1.7	2.0	1.4	1.9	0.8	1.5	1.8	1.6
204	Leukemia	4.6	6.0	4.6	5.0	4.1	4.5	4.5	3.1	6.3	2.9
201	Lymphogranulomatosis, Hodgkin	1.4	2.1	1.8	1.4	1.4	1.6	0.8	1.9	2.4	1.6
200, 202, 203, 205	Denmark incl.: All sarcomas of various sites except bones and joints.	5.9	6.8	5.9	5.7	4.9	4.9	4.0	5.0	5.1	5.1
	All malignant neoplasms	263.7	272.5	254.4	241.5	212.3	208.6	246.8	243.1	333.4	293.0

*††*See* footnotes, table 1.

1) *Esophageal cancer.*—No certain differences in rates are shown.

2) *Gastric cancer.*—White men and women in the United States show a lower incidence of gastric cancer than the Copenhagen population. Nonwhites show rates approximately equal to those for the corresponding sex in Copenhagen, and women in all categories show lower rates than men.

3) *Cancer of large intestine.*—It is in the nature of this diagnosis that its delimitation from cancer of the rectum will be open to discussion. It appears that the sum of the rates of these two cancers for white men is the same in the two countries. White women show considerably higher rates in the United States than in Copenhagen, largely due to a difference for cancer of the large intestine.

4) *Liver cancer.*—The incidence is almost the same for men and women in all three categories. Cancer of biliary passages is fairly rare for nonwhites of both sexes, while pancreatic carcinoma on the whole seems more frequent in the 10 cities of the United States than in Copenhagen.

Respiratory System

Lung.—This is difficult to comment on because, due to the rapid increase in rates for lung cancer, even a small difference in date of observation will influence comparisons materially. However, even if allowance is made for this factor there still seems to be some difference, the rates of Copenhagen men being slightly higher than those in the United States cities. For women, differences are negligible.

Larynx and trachea.—The rates are considerably higher for United States men than for Danish men, irrespective of the color of the skin. For women, differences are negligible.

Breast

While white women in United States cities show higher rates than nonwhites, the Danish women show approximately the same rates as nonwhites.

Genital Organs

1) *Uterus.*—Judging from total figures, we find that nonwhite women show more uterine cancer than white women in cities of the United States and Copenhagen. However, the relatively high proportion of unspecified cases in United States cities makes further analysis difficult, although it may be said that cervical carcinoma is much more frequent among nonwhites than among whites.

2) *Other female genital organs.*—The data for other female genital organs probably do not allow any detailed analysis because the diagnostic facilities in each city have a considerable influence on the number of cases diagnosed.

3) *Male genital organs.*—High rates for nonwhites are shown, apparently due to prostatic cancer, which is more frequently diagnosed in United States cities than in Copenhagen.

Cancer of Urinary Organs

Rates have increased among men in Copenhagen (*9*) due to a sharp rise in bladder tumors, and the rate for this city is now in line with that for white males in the 10 cities of the United States. However, it should be kept in mind that Danish data for this site include papillomas. A recent comparison of mortality for bladder tumors in various countries (*15*) showed, on the whole, identical experience for the United States and Denmark. Attention should be drawn to the fact that Danish incidence figures for kidney—which have been constant since 1942 (*9*)—are higher than United States figures for both sexes among whites and nonwhites.

Cancer of Skin

High figures for United States whites of both sexes are shown and low figures for nonwhites, while Danish figures are intermediate and are consistent with earlier results reported by Dorn.

Tumors of Brain, Nervous System, and Other Sites

A detailed analysis cannot be made because too few occur and the cases are too influenced by differences in diagnostic means.

The remaining categories do not seem to call for discussion.

Summary

Data are given on recent cancer incidence in Denmark, which have been adjusted for age with the same standard population employed in the presentation of experience in the United States. The Danish results are compared with those from the cancer morbidity survey in 10 cities of the United States.

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Effects of Lytic Agents on Plasma Membrane of Ehrlich Ascites-Tumor Cells and Mouse Erythrocytes [1, 2]

LESLIE R. BENNETT *and* FRANK E. CONNON,[3] *Department of Radiology and the Atomic Energy Project, School of Medicine, University of California, Los Angles, California*

The effects of various agents upon the plasma membrane of Ehrlich ascites-tumor cells and mouse erythrocytes *in vitro* are reported in this paper. Earlier experiments (*1*, *2*) demonstrated that oleic acid, a constituent of a microsomal sediment from small intestines of mice and rats, strongly depressed oxygen uptake of Gardner lymphosarcoma 6C3HED, Yoshida sarcoma, and Ehrlich ascites-tumor cells. The degree of respiratory depression was closely correlated with cell-membrane damage of a lytic type, which will be discussed.

Fatty acids are known to possess hemolytic properties (*3*). In common with other lytic agents, fatty acids act mainly upon the lipoids of the plasma membrane, with destruction of the membrane and subsequent release of cytoplasm (*4*). Our *in vitro* technique indicated that tumor-cell membranes acted upon by oleic acid were damaged in a manner similar to the process of hemolysis. This observation led to a comparative study of the effects of a number of compounds on the plasma membrane of erythrocytes and Ehrlich tumor cells. Early experiments were confined to study of substances with known hemolytic activity; later, compounds structurally or otherwise related to such substances were included; finally, compounds unrelated to hemolytic agents were tested. A standardized procedure was developed for determining the extent of membrane damage *in vitro*, with mouse erythrocytes and tumor cells. The method permitted observations of leukocytes also, since these were present both in blood and ascitic fluid.

Experimental

Procedure.—Seven-day-old Ehrlich ascites-tumor cells were used in all experiments. The cells were washed 3 times with calcium-free Krebs-

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[2] This study was performed under contract between The Atomic Energy Commission and The University of California at Los Angeles, and was supported in part by grants from the University of California cancer research funds and the California Institute for Cancer Research.

[3] Development of the procedure used in these experiments is largely due to the demonstration by Dr. Riojun Kinosita of similar phenomena in smears of Yoshida sarcoma cells.

The authors express their appreciation to Dr. Andrew H. Dowdy for valuable suggestions and criticism. Thanks are extended to Dr. James Mead and Dr. Judd Nevenzel for generous assistance, to Dr. Ralph Nussbaum and Dr. Richard Riley for aid in identification of some of the compounds used in these experiments, to Mr. Francis Bishop for electron-microscopy studies, and to Mrs. Patricia Littlewood for phase-microscope aspects of the present work.

Ringer phosphate buffer, *p*H 7.4, and a 15 percent suspension in buffer was prepared. To this was added an equal volume of a 10 percent suspension of washed, normal mouse erythrocytes. These concentrations of cells were used arbitrarily as a standard. One cc. of the combined suspension was pipetted into 10 cc. tubes containing graded amounts of the test substances. Final volumes were adjusted to 3.0 cc. with buffer. Control tubes contained only buffer and cells. In experiments where serum was included to test its effects upon the lytic process, 0.25 cc. of normal mouse serum replaced an equal amount of buffer solution in the system. In most instances, compounds were added to the tubes at 4 levels: 0.25, 0.5, 0.75, and 1.0 mg. Since the total volume of solution in each tube was 3.0 cc., the concentration per cc. was one third of these amounts and is so indicated in the tables. Test materials that were insoluble in buffer were dissolved in a minimal amount of ethanol and then suspended in buffer. Ethanol alone, in the amounts required, was shown to be nonlytic to tumor and blood cells.

The tubes were immersed in a water bath at 37.5° C. for 1 hour and agitated gently at 10-minute intervals to maintain the cells in a suspended state. Following incubation, the tubes were centrifuged and the supernatant examined for hemolysis which was graded 4+ when complete and 0 when absent. The degree of damage to tumor-cell membranes was judged according to standards as follows: (*a*) *Intact cells.* Such cells exhibited no evidence of cell membrane, cytoplasmic, or nuclear damage and were essentially "normal" in appearance. (*b*) *Partially destroyed cells.* This classification included cells that showed evidence of cell-membrane rupture, bleb formation, and/or extensive vacuolation and shredding of the cytoplasm. (*c*) *Naked nuclei.* The cytoplasm of cells so classified was stripped away, leaving only the nucleus, with the membrane intact. Smears of nuclear material often encountered in preparations from higher levels of concentration of lytic agents were not included in cell counts. Giemsa stain was found to be most satisfactory for use in the preparation of smears for microscopic examination since the 3 types of cell were easily differentiated by color and identifiable even under low magnification. Smears made directly from aspirated ascitic fluid contained all the cell types described, but as would be expected, due to minimal handling, the number of intact cells was relatively high in comparison to the counts found in smears made from experimental suspensions. Figure 1 shows a portion of a typical smear.

Method of counting.—Tubes at each level of concentration of the material tested were run in duplicate in early determinations. Counts from duplicate tubes were sufficiently comparable to permit confidence in values from single tubes in later experiments. Duplicate smears were prepared from the tubes and counts of from 100 to 1,000 cells were made under high-dry or oil-immersion objective (550 to 1,250 magnifications). Since mechanical breakage due to smearing was recognized as a problem in the procedure, counts were made of fields chosen at random over the entire surface of the slide. Average values for 2 to 4 individual experi-

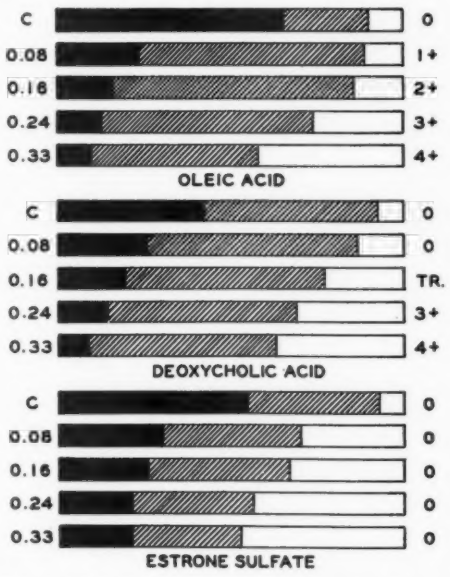
ments involving cells from different animals were calculated and the data are presented in the tables.

Phase-microscope study.—The possibility of drawing erroneous conclusions from smears was recognized, and phase-microscope observations of living cells in buffer containing lytic substances were made. Representative fields were photographed.

Compounds tested.—Many of the substances tested were supplied commercially. Others were prepared in the laboratory by standard procedures or synthesized elsewhere for our use. Extracts of third-trimester human pregnancy urine were made by a modification of the method of Cohen, Marrian, and Odell (*5*). The extracted material contained a mixture of conjugated glucuronides identified by the procedure of Maughan, Evelyn, and Browne (*6*).

Results

Experimental data are presented in the tables. Limitations of space dictated the inclusion of data for the 0.33 mg. level of concentration only. Complete data at the 4 levels usually employed showed, in all cases involving actively lytic substances, a decrease in number of intact cells with increasing concentration and a corresponding increase in number of partially destroyed cells and naked nuclei. Text-figure 1 indicates this



TEXT-FIGURE 1.—Effects of lytic-agent concentration on cell counts. *Fully shaded areas* indicate percent of intact cells; *partly shaded areas*, partially destroyed cells; *unshaded areas*, naked nuclei. C=Control counts. Figures at *left* are the concentrations of lytic agents in mg. per cc. Numbers at *right*, degree of hemolysis (0, none; 4+ complete). Graphs drawn from average values for 4 experiments in each case.

for 3 representative compounds, oleic acid, deoxycholic acid, and estrone sulfate.

Data for lytic activity of a series of fatty acids are given in table 1. At the concentrations used, it was apparent that membrane-damaging activity became more pronounced as the number of carbon atoms in the chain increased, and that hemolytic properties were encountered only in the fatty acids of greater chain length. Hemolytic activity was reduced by normal mouse serum in the system, but only minimal effects on tumor-cell membranes were noted. Iodination of oleic acid did not change the lytic properties of the parent compound.

TABLE 1.—*Cytolysis in the presence of fatty acids*

Compound (*0.33 mg.*)	Tumor-cell count			
	Hemolysis	Intact (*percent*)	Partially destroyed (*percent*)	Naked nuclei (*percent*)
Control	0	52	46	2
Butyric acid	0	43	53	4
Valeric acid	0	40	55	5
Caproic acid	0	30	60	10
Heptanoic acid	0	30	52	18
Control	0	59	36	5
Octanoic acid	0	18	56	26
Octanoic acid + serum	0	17	58	25
Control	0	58	38	4
Capric acid	0	20	60	20
Capric acid + serum	0	22	59	19
Lauric acid*	4+	18	57	25
Lauric acid + serum*	2+	10	60	30
Control	0	65	25	10
Oleic acid*	4+	10	48	42
Control	0	68	26	6
Oleic acid + serum*	1–2+	7	43	50
Control	0	44	46	10
Iodinated oleic acid	4+	11	43	46

*Insoluble in buffer; used as suspension.

With the exception of the sodium salt of dehydrocholic acid, all the bile acids were weakly to strongly hemolytic. Normal serum reduced the hemolytic effects to some extent, but did not greatly affect the damaging properties to tumor cells (table 2).

The nature of the damage to the tumor cells in the presence of sex steroids was typically lytic. The majority of the sex steroids studied (table 3) were nonhemolytic and, in general, on a weight basis, they were slightly less damaging to tumor cells than were the bile acids investigated. Evidence for estrone acetate oxim and its Girard t reagent derivative suggests that the lytic action was not hormonal since these compounds are nonestrogenic. Butanol extracts of third-trimester human pregnancy urine were found to contain several lytic substances. Such extracts, puri-

TABLE 2.—*Cytolysis in the presence of bile acids and bile-acid derivatives*

Compound (*0.33 mg.*)	Tumor-cell count			
	Hemolysis	Intact (*percent*)	Partially destroyed (*percent*)	Naked nuclei (*percent*)
Control	0	49	44	7
Bile salts	trace	17	50	33
Control	0	42	50	8
Deoxycholic acid*	4+	9	54	37
Control	0	51	42	7
Deoxycholic acid + serum*	<1+	7	34	59
Control	0	51	40	9
Methyl ester, deoxycholic acid*	4+	7	34	59
Control	0	32	63	5
Sodium dehydrocholate	0	42	51	7
Control	0	59	35	6
3-Octanoyl deoxycholic acid*	3–4+	9	55	36
Control	0	55	37	8
3-Octanoyl deoxycholic acid + serum*	<1+	6	46	48

*Insoluble in buffer; used as suspension.

fied according to the procedure of Cohen *et al.* (*5*) and of Venning (*7*) yielded fractions corresponding to pregnanediol and estriol glucuronides. The fractions exhibited lytic activity comparable to commercially prepared glucuronides of these steroids. In addition to these compounds, there was evidence of other unidentified lytic substances in the urinary extracts.

For comparative purposes, tumor-cell suspensions were exposed to known mitotic inhibitors (urethan, aminopterins, and X irradiation; tables 4 and 5). The data for urethan and the aminopterins indicate a slight decrease in the number of intact cells and little increase in the number of naked nuclei as compared with control values. Typical concentration effects were absent, *i.e.*, the cell counts did not vary with changes in concentration of the agents. After exposure to X ray, cell suspensions were incubated for 1 hour at 37.5° C. Little or no damage to the plasma membranes was noted. However, both cytoplasm and nucleoplasm exhibited extensive vacuolation, indicating destructive effects not found after exposure to lytic substances, but consistent with known effects of radiation.

Tumor cells suspended in distilled water for 1 hour exhibited osmotic changes. Increase in total diameter was noted, and occasional bursting was observed. The plasma membranes were not damaged in a manner suggesting local destruction, as was repeatedly observed in the presence of lytic agents. It seems apparent, therefore, that the increased fragility of the tumor-cell plasma membrane was not due entirely, if at all, to osmotic effects.

TABLE 3.—*Cytolysis in the presence of various steroids and steroid derivatives*

Compound (*0.33 mg.*)	Tumor-cell count			
	Hemolysis	Intact (*percent*)	Partially destroyed (*percent*)	Naked nuclei (*percent*)
Control	0	55	38	7
Estrone sulfate	0	23	30	47
Control	0	60	30	10
Estrone sulfate + serum	0	21	44	35
Control	0	60	30	10
Estrone acetate*	4+	8	45	47
Estrone acetate + serum*	4+	30	28	42
Estrone acetate oxim*	4+	17	39	44
Estrone acetate oxim + serum*	0	22	50	38
Control	0	52	41	7
Estrone acetate oxim + Girard t reagent*	0	12	48	40
Control	0	69	22	9
Testosterone*	0	5	58	37
Progesterone*	0	6	67	27
Estradiol	0	23	49	28
Control	0	59	35	6
Diethylstilbestrol diphosphate	0	11	61	28
Control	0	58	35	7
Pregnanediol*	0	17	54	29
Pregnanediol glucuronide*	0	30	45	25
Control	0	59	35	6
Estriol*	0	10	61	29
Control	0	48	42	10
Estriol glucuronide*	0	23	46	31
Control	0	64	32	4
Deoxycorticosterone acetate*	0	62	34	4
Control	0	59	34	7
Hydrocortisone*	0	57	35	8

*Insoluble in buffer; used as suspension.

Phase-microscope studies indicated that cell destruction observed in smears was not caused entirely by the mechanical effects of smearing. Figures 3 to 5 show extensive damage to cells in the living state. Blister formation, raising of the plasma membrane, and vacuolation were all noted except in control suspensions (fig. 2). Cells exhibiting these effects could be expected to be much more fragile and subject to mechanical breakage than undamaged cells. Damage that occurred before smearing was reflected in increased numbers of partially destroyed cells and naked nuclei in smears.

Data for the effects upon leukocytes of the lytic agents investigated have not been tabulated. Examination of smears indicated that, with practically no exceptions, these cells were not damaged by agents which exhibited marked effects upon tumor cells unless hemolysis was observed also.

TABLE 4.—*Lack of cytolysis following exposure to mitotic inhibitors (urethan, 4-aminopteroylaspartic acid and 4-aminopteroylglutamic acid)*

Compound (*0.33 mg.*)	Tumor-cell count			
	Hemolysis	Intact (*percent*)	Partially destroyed (*percent*)	Naked nuclei (*percent*)
Control	0	58	33	9
Urethan	0	50	39	11
Control	0	49	43	8
4-Aminopteroylaspartic acid	0	47	44	9
4-Aminopteroylglutamic acid	0	42	49	9

TABLE 5.—*Lack of cytolysis in Ehrlich ascites tumor cells after X irradiation*

Dose (*r*)	Intact (*percent*)	Partially destroyed (*percent*)	Naked nuclei (*percent*)
Control	43	48	9
1,000	46	44	10
2,000	42	50	8
3,000	44	46	10

Discussion

It is generally accepted that the plasma membrane is a lipoid-protein combination, but the orientation of these elements is still debated [*cf.* Danielli (*4*), Parpart and Ballentine (*8*), Hillier and Hoffmann (*9*)]. Despite lack of agreement on fundamental arrangement, evidence has accumulated that suggests differences in structure, composition, and function of the malignant cell membrane as compared with that of the normal cell. Cowdry (*10*) has pointed out, however, that observed differences between malignant cells and their normal cells of origin do not apply in all instances.

Electron microscopy has provided some information of rather limited nature. Gessler *et al.* (*11*) found that the nuclear and plasma membranes of cancer cells often were broken and the cytoplasm fibrillar in character. This could be interpreted as indicating increased fragility of the cells and their components, or as due to technique employed, since similar findings have not been emphasized in later work. Luse and Reagan (*12*) observed a "complicated plasma membrane" in human cancer cells, with numerous distinct microvilli covering the surfaces. Selby *et al.* (*13*) found similar surface protuberances in a variety of cancer cells, and they have been observed in electron photomicrographs of Ehrlich ascites-tumor cells and Gardner lymphosarcoma cells in our laboratory. Enterline and Coman (*14*) suggested that motile amoeboid processes seen in motion pictures of cancer cells, together with lowered adhesiveness and low calcium content might be responsible for the invasiveness of cancer cells.

Coman and Anderson (*15*) induced malignant changes in rabbit epithelium and measured the diameters of chromium-carbon replicas of cell

surface plaques. Whereas such plaques were 30 to 60 Å in diameter in normal epithelial cells, they were 30 to 300 Å in diameter in cells showing malignant changes. The pores between the plaques probably vary correspondingly and might be expected to alter permeability characteristics of the malignant cells.

Differences in permeability of normal and malignant cells have, in fact, been reported. Ludford (*16*) observed increased permeability to fat-soluble dyes in cancer cells. Lucké and Parpart (*17*) found that cancer cells were less rapidly permeable to polyhydric alcohols than were normal mouse erythrocytes. Low calcium content of cancer cell membranes may affect permeability [Stern and Willheim, (*18*)] and microincineration studies have indicated a decreased calcium storage capacity in cancer cells [Scott, (*19*); Lansing *et al.*, (*20*); DeLong *et al.*, (*21*)].

The higher fat content of the cancer cell membrane has been emphasized (*16*). This may account for the differential lytic effects of the fatty acids in our experiments, for sodium and potassium salts of these compounds are known to attack the lipid portion of the cell membrane (*4*). The lytic action of some of the sex steroids tested is less easily explained, since the adrenal steroids were inactive. The high lytic activity of estrone acetate oxim and its Girard t reagent derivative suggests that steric configuration is of primary importance in determining whether or not steroid compounds are lytic to erythrocytes, tumor cells, or both. The possible importance of steric factors is also seen in sodium dehydrocholate, which is inactive, while deoxycholic acid is strongly destructive to tumor cells and erythrocytes.

The sex steroid glucuronides are of special interest, since they are usually looked upon as detoxification or excretion products of hormones (*7*). Observations of membrane-damaging capacity in our experiments suggest that some of the conjugated glucuronides may have heretofore unsuspected biological significance.

The work of Leonard and Rutstein (*22*) with unfertilized eggs of the marine worm *Chaetopterus pergamentaceus* is of particular interest in relation to our studies. Eggs exposed to progesterone, deoxycorticosterone, and testosterone showed morphologic changes corresponding closely to those observed in Ehrlich tumor cells. Of these compounds, only deoxycorticosterone showed a lack of damaging effect upon Ehrlich tumor-cell membranes. Hydrocortisone did not cause morphologic changes in either *Chaetopterus* eggs or Ehrlich tumor cells. Leonard and Rutstein employed different concentrations of agents, lower temperatures, and varying lengths of exposure compared to those in our studies. Nevertheless, the effects noted were strikingly similar and suggest that the plasma membranes of undifferentiated and dedifferentiated cells may react to lytic agents in a comparable manner.

The cell-surface phenomena observed in our studies suggest that malignant cells of at least one type may react in a manner different from normal cells in the presence of lytic agents. This may reflect fundamental differences in structure and/or function of the cancer cell membrane as

compared to normal cells. The data suggest also that certain agents have rather specific effects upon the cancer cell membrane.

Summary

1) An *in vitro* procedure for the comparative study of lysis of mouse erythrocytes and Ehrlich ascites-tumor cells is described.

2) A series of compounds including fatty acids, bile acids, and steroids was tested. Comparative effects of mitotic inhibitors and X rays were studied also. Data for lytic effects of these agents are presented.

3) Among the materials studied, a number of compounds were found that seemed to possess selective lytic properties for cancer cells *in vitro* in the absence of similar lytic effects upon mouse erythrocytes and leukocytes.

4) The significance of the phenomena observed in these experiments in relation to previous studies is discussed.

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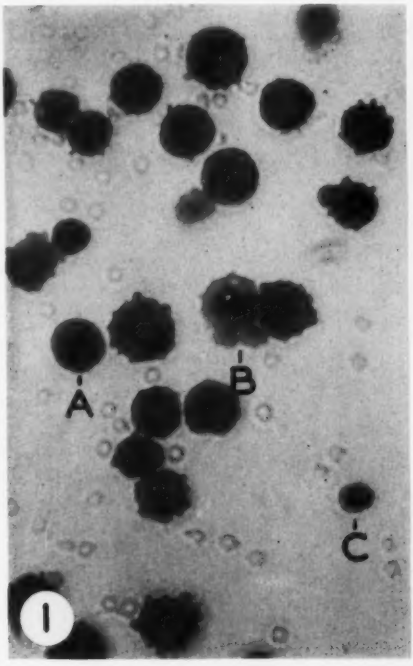
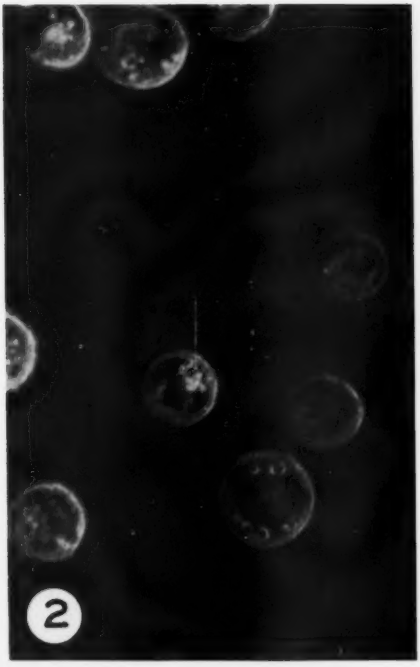
PLATE 85

FIGURE 1.—Typical smear of Ehrlich tumor cells after exposure to oleic acid (0.16 mg./cc.) for 1 hour at 37.5° C. Cells were centrifuged, resuspended in Krebs-Ringer phosphate buffer, *p*H 7.4, smeared, and stained with Giemsa stain. Hemolysis was rated 2+. A=Intact cell with undamaged plasma membrane. B=Partially destroyed cell. C=Naked nucleus. Other cells show varying degrees of lytic damage.

FIGURE 2.—Phase photomicrograph of a control suspension of Ehrlich tumor cells in Krebs-Ringer phosphate buffer, *p*H 7.4. The suspension was incubated at 37.5° C. for 1 hour. Erythrocytes were not added. Bright-light contrast.

FIGURE 3.—Effect of oleic acid on Ehrlich tumor cells. Concentration, 0.33 mg. per cc., exposure time 1 hour at 37.5° C. Hemolysis was rated 3 to 4+. Tumor-cell plasma membranes show "blistering" and extensive cytoplasmic damage. Bright-light contrast.





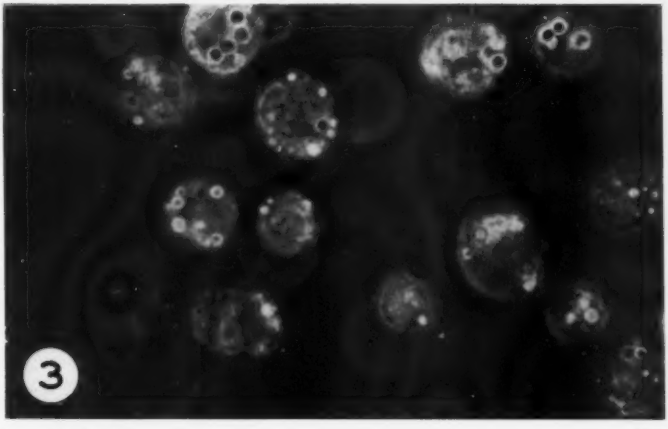


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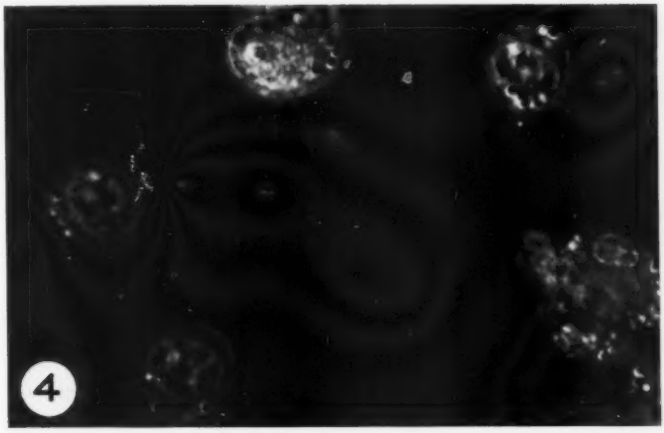
PLATE 86

FIGURE 4.—Damage to Ehrlich tumor cells exposed to deoxycholic acid (0.33 mg./cc.) for 1 hour at 37.5° C. Erythrocytes added at the beginning of incubation were completely hemolyzed. Tumor cells show typical morphologic changes observed in the presence of this and other lytic agents. Dark-medium contrast.

FIGURE 5.—Effects of estrone sulfate (0.33 mg./cc.) on Ehrlich tumor cells and mouse erythrocytes. The latter show no morphologic changes, but tumor cells exhibit varying degrees of damage. Bright-light contrast.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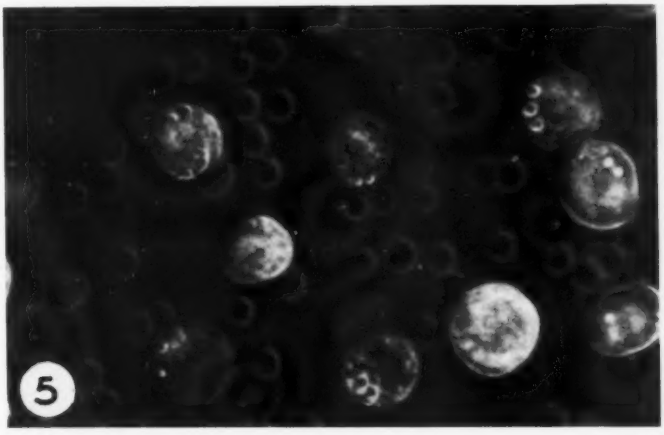
5

Sex as a Factor in Skin-Tumor Metastasis[1,2,3]

WILLIAM E. POEL,[4] *Laboratory for Experimental Carcinogenesis, Department of Occupational Health, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania*

Metastasis has been observed in this laboratory as a frequent occurrence among mice with chemically induced squamous-cell carcinomas of the interscapular and dorsal thoracic skin. A relationship between the frequency of metastasis from these tumors and the sex of the host was indicated by the preponderance of metastasis among the tumor-bearing females. The observed difference in incidence between the sexes has suggested the hypothesis that metastasis from squamous-cell carcinoma may be related, in part, to endogenous sex-hormone levels.

Experimental Procedure

Skin-tumor induction was effected by applications of a carcinogenic light creosote oil, as a 20, 40, or 80 percent solution in toluene, or by application of benzo[*a*]pyrene (3,4-benzpyrene), as a 0.25 or 1.25 percent solution in toluene. These solutions, applied repeatedly to the shaved interscapular skin of male and female DBA/2 mice, were found to produce epidermoid carcinomas consistently (*1*). In the present study, approximately 0.009 cc. of one of the above solutions was applied dropwise, 3 times a week, with a calibrated, blunted hypodermic needle dropper, to the interscapular skin of DBA/2 and C57L mice until malignant tumors were induced. Malignancy was estimated grossly by the presence of a skin tumor, approximately 6 to 8 mm. in diameter, possessing a subcutaneously thickened base and a rolled or elevated peripheral margin. After exposures were stopped, growth of the tumor was allowed to proceed until terminated by the natural death of the host or until the animal became obviously moribund, at which time it was sacrificed. Control groups, consisting of mice of the same strain, age, and sex, were treated concurrently with toluene. Representative histologic sections were made of the principal organs and pathologic tissues of each mouse,

[1] Received for publication, March 11, 1957.

[2] This work was supported by grant CS-9187 from the Field Investigations and Demonstrations Branch, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and the Martha O. Freas #3 grant-in-aid, American Cancer Society, Inc.

[3] Delivered in part at the meetings of the American Association for Cancer Research, Chicago, April 14, 1957.

[4] The author is indebted to Nona Karten, Evelyn Peters, Dolores Stanton, Henry Wade, and John E. Thomas for their technical assistance in this study.

except for the skull and skeletal system. Multiple sections were made of tissues that showed gross signs of possible metastasis.

Results

Tumor Induction

No papilloma or skin tumor of any type was observed among the toluene-treated controls.

The induced neoplasms among the benzopyrene or creosote-oil treated groups were hyperkeratotic papillomas initially, which on continued exposure became squamous-cell carcinomas. The papilloma appearance time, or exposure duration before grossly apparent papillomas[5] were induced for each strain at each exposure level, is shown in table 1. From this table the following comparisons can be made (cols. 1–6):

Sex.—A comparison of the sexes shows no consistent difference in average papilloma appearance time, although the impression gained in the laboratory was that the earliest papillomas appeared first among the males (cols. 9, 10).

Strain.—An earlier appearance of papillomas among the C57L mice, as compared with the DBA/2, is seen at all exposure levels below the highest dosage of benzopyrene applied (100 μg. per drop of 1.25% solution). These results parallel a previous finding of greater susceptibility to tumor induction with benzopyrene on the part of the related C57BL strain in comparison with the DBA/2 (*2*).

Dose-response relations.—All mice effectively exposed to the 80 percent solution of creosote oil, or to the 2 dose levels of benzopyrene developed epidermoid carcinomas. For both carcinogens, the higher dosage was associated with a relatively shorter papilloma appearance time. These data confirm the observation that, within limits, skin-tumor appearance time is an inverse function of the dose of carcinogen applied repeatedly (*3*). The difference between the DBA/2 and C57L strains in their neoplastic response at all but the highest dose level of carcinogen employed supports the impressions of other investigators that subtle biologic differences are overwhelmed by large doses of a carcinogen, and that these differences can best be studied with the smallest possible amount found to be effective (*4*, *5*).

Exposure Duration Preceding Grossly Apparent Epidermoid Carcinomas

Table 2 shows the exposure periods that preceded malignant-appearing skin tumors of comparable size[6] at the various exposure levels employed. These data indicate shorter exposure periods or a more rapid development in size of the carcinomas induced with the higher dose levels. The dosage, or intensity of the intermittent carcinogenic exposure, together with duration of exposure (*6*), thus appear to govern the rates of papilloma growth

[5] Approximately 0.5 to 2 mm. in diameter when first recorded.

[6] Approximately 6 to 8 mm. in diameter, with subcutaneously thickened base and rolled margins.

TABLE 1.—*Papilloma appearance time in relation to strain, dose of carcinogen, and metastasis, in mice with induced epidermoid carcinomas*

Strain of mice	Agent (*percent in toluene*)	No. with epidermoid carcinoma		Papilloma appearance time									
				Mice with primary interscapular carcinoma						Mice with metastasis			
				Weeks of exposure						No. with metastasis		Weeks of exposure Papilloma appearance time	
				Median		Mean		Range					
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
DBA/2	1.25 Benzopyrene	12	10	16	16	15. 5	15. 4	12–19	12–20	2	4	16, 19	12, 14, 16, 20
	0.25 Benzopyrene	13	12	25	28	26. 8	26. 4	16–38	19–34	1	2	22	28, 30
	80 Light creosote oil	10	10	36	34	34. 5	35. 7	22–41	25–48	0	2	—	34, 48
	40 Light creosote oil	12	13	44	34	45. 0	35. 5	25–62	25–61	1	3	38	28, 34, 38
	20 Light creosote oil	6	7	44	61	43. 3	65. 4	34–56	56–81	1	0	46	—
C57L	1.25 Benzopyrene	7	7	16	18	14. 6	16. 9	8–18	8–21	0	2	—	8, 21
	0.25 Benzopyrene	8	8	14	18	15. 0	18. 5	11–21	14–24	1	2	16	18, 21
	80 Light creosote oil	10	7	27	22	26. 2	24. 6	18–34	21–35	0	2	—	21, 28
	40 Light creosote oil	2	4	—	—	—	—	25	20–33	0	1	—	33
	20 Light creosote oil	5	7	31	27	28. 4	29. 3	18–37	22–43	0	2	—	24, 31
Total mice		85	85							6	20		

TABLE 2.—*Duration of exposure until induced tumors appeared grossly malignant, in relation to strain, dose of carcinogen, and presence of metastasis*

Strain of mice	Agent (*percent in toluene*)	No. with epidermoid carcinoma		Duration of exposure until induced tumors were grossly malignant									
				Mice with primary interscapular carcinoma						Mice with metastasis			
				Weeks of exposure						No. with metastasis		Weeks of exposure until primary tumors were grossly malignant	
		♂	♀	Median		Mean		Range					
				♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
DBA/2	1. 25 Benzopyrene	12	10	20	21	19. 5	20. 0	19–20	16–22	2	4	19, 19	16, 19, 22, 22
	0. 25 Benzopyrene	13	12	32	33	34. 0	35. 1	22–42	32–41	1	2	28	32, 36
	80 Light creosote oil	10	10	44	47	44. 3	45. 4	34–57	28–56	0	2	—	51, 51
	40 Light creosote oil	12	13	55	43	55. 7	49. 0	48–66	43–63	1	3	48	43, 43, 43
	20 Light creosote oil	6	7	58	77	57. 8	75. 7	56–59	61–87	1	0	56	—
C57L	1. 25 Benzopyrene	7	7	21	21	19. 1	18. 6	14–22	11–21	0	2	—	11, 21
	0. 25 Benzopyrene	8	8	22	22	24. 2	24. 0	21–39	19–33	1	2	21	21, 27
	80 Light creosote oil	10	7	42	34	39. 8	33. 9	27–43	21–42	0	2	—	34, 40
	40 Light creosote oil	2	4	—	—	—	—	27, 27	27, 33, 37, 38	0	1	—	38
	20 Light creosote oil	5	7	51	37	48. 2	37. 7	41–53	27–43	0	2	—	34, 37

and transformation into invasive malignant neoplasms, as they apparently regulate the initial papilloma induction time and papilloma incidence.

Sex and strain differences.—The absence of a significant sex difference in duration of exposure before grossly malignant tumors developed (table 2) parallels the observation that there was no consistent sex difference in papilloma appearance time after chronic intermittent carcinogenic exposures (table 1). It appears that sex in this experiment was not a significant factor in modifying the host's initial susceptibility to a topical carcinogenic stimulus. However, the strain difference, noted as a host factor modifying susceptibility to papilloma induction, is seen in table 2 to apply also to the rapidity with which the induced papillomas were converted to malignant growths.

Frequency of Metastasis

The occurrence of metastasis among tumor-bearing mice treated with light creosote oil has already been reported (*1*). In the present study, metastasis at *post-mortem* examination was found in 7 percent of all males and 23 percent of all females with induced tumors. These proportions, for skin-tumor metastases, are higher than have heretofore been described in the literature (*7*, *8*). It is possible that the greater frequency now reported is related to the procedure of keeping tumor-bearing animals until their natural death instead of sacrificing them prematurely after development of the primary neoplasm.

The sites observed with metastases to date have been the cervical and axillary lymph nodes and the lungs. Involvement of these sites in mice with primary squamous-cell carcinomas of the dorsal thoracic and cervical skin suggests that the same type of tumor induced at different specified skin areas might provide an experimental technique for the study of spontaneous metastasis, without surgical or pharmacologic alteration of the host.[7]

Sex of the Host and Metastasis

A difference in incidence of metastasis appears to be associated with the sex of the tumor-bearing host, as shown in tables 1 to 3. This was a consistent finding in 9 of the 10 different exposure groups listed for both strains. The following two analyses indicate that the higher incidence among the females was probably not due to chance.[8]

1) For each of the 10 exposure groups tested of both strains, the difference between the proportion of males and the proportion of females with metastases was calculated. These differences were obtained from the arc-sin transformation of the proportions in an attempt to "normalize" the distribution of the variable.

The differences were summed and the t test (for paired observations) was used to determine how much the mean of the 10 differences deviated

[7] In contrast with the effects obtained by cortisone treatment of a tumor-bearing host, tumor transplantation techniques, and injection of tumor-cell suspensions into the blood-vascular or lymphatic systems (*7*, *9*, *10*).

[8] Analyses were made by Dr. C. C. Li and Dr. A. Ciocco, Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, to whom the author is deeply indebted.

TABLE 3.—*Life span of mice with induced epidermoid carcinomas, in relation to dose of carcinogen and presence of metastasis*

Strain of mice	Agent (*percent in toluene*)	No. with epidermoid carcinoma		Life span									
				Mice with primary interscapular carcinoma (*life span in weeks after initial exposure*)						Mice with metastasis			
				Median		Mean		Range		No. with metastasis		Life span (*weeks after initial exposure*)	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
DBA/2	1.25 Benzopyrene	12	10	30	32	30. 2	32. 4	24–38	32–43	2	4	31, 38	31, 31, 31, 36
	0.25 Benzopyrene	13	12	44	46	44. 3	46. 4	33–51	37–56	1	2	33	41, 51
	80 Light creosote oil	10	10	48	54	48. 8	49. 9	37–61	34–60	0	2	—	53, 57
	40 Light creosote oil	12	13	60	57	59. 3	58. 2	49–69	46–74	1	3	65	49, 52, 65
	20 Light creosote oil	6	7	59	77	59. 2	77. 3	56–64	61–89	1	0	64	—
C57L	1.25 Benzopyrene	7	7	30	31	29. 1	30. 7	19–39	22–43	0	2	—	31, 43
	0.25 Benzopyrene	8	8	40	37	37. 0	35. 9	27–43	21–44	1	2	41	35, 37
	80 Light creosote oil	10	7	42	41	40. 5	39. 9	34–43	36–42	0	2	—	40, 41
	40 Light creosote oil	2	4	—	—	—	—	42	27–58	0	1	—	58
	20 Light creosote oil	5	7	51	43	49. 4	45. 9	41–56	37–58	0	2	—	43, 43

from 0. The assumption tested was, that if chance alone were operating, the mean of the differences should be 0. The t was 3.1: thus, with 9 degrees of freedom, the probability (P) that the observed mean of the differences could have occurred by chance if the true difference were 0 was found to be very small, *i.e.*, $0.01 < P < 0.02$ (table 4).

TABLE 4.—*Analysis of data on mice with metastasis among tumor-bearing mice, according to sex, strain, and exposure*

Strain of mice	Agent (*percent in toluene*)	Mice with metastasis among mice with primary carcinomas		Arc-sin transformation of fractions of mice with metastasis, according to sex, strain, and exposure		
				Angle in degrees		Difference between sexes x
		♂	♀	♂	♀	
DBA/2....	1.25 Benzopyrene....	2/12	4/10	24. 1	39. 2	+15. 1
	0.25 Benzopyrene....	1/13	2/12	16. 1	24. 1	+ 8. 0
	80 Light cresote oil..	0/10	2/10	0	26. 6	+26. 6
	40 Light cresote oil..	1/12	3/13	16. 8	28. 7	+11. 9
	20 Light cresote oil..	1/6	0/7	24. 1	0	−24. 1
C57L.....	1.25 Benzopyrene....	0/7	2/7	0	32. 3	+32. 2
	0.25 Benzopyrene....	1/8	2/8	20. 7	30. 0	+ 9. 3
	80 Light cresote oil..	0/10	2/7	0	32. 3	+32. 3
	40 Light cresote oil..	0/2	1/4	0	30. 0	+30. 0
	20 Light cresote oil..	0/5	2/7	0	32. 3	+32. 3
Total.....		6/85	20/85	101. 8	275. 5	+173. 7
				t=3.1;		$0.01 < P < 0.02$

2) Since findings based on small numbers must always be accepted with caution, an analysis of the pooled data on the 10 experimental groups was made without regard for strain differences or dose level of exposure (table 5).

TABLE 5.—*Pooled data from table 4*

	Mice with primary carcinoma		Total
	Males	Females	
With metastasis.....	6	20	26
Without metastasis.....	79	65	144
Total.....	85	85	170

Chi square for this tabulation is 8.9 with 1 degree of freedom. It is found that $0.001 < P < 0.01$. The tentative assumption on which the latter analysis is based is that neither dose nor strain of host affect the over-all sex difference in occurrence of metastasis. Adequate data in support of

this hypothesis are not yet ready for publication, and the former, more conservative analysis, is preferred for the present. However, both analyses support the conclusion that the sex difference in the occurrence of metastasis is real.[9]

Metastasis and Susceptibility to Primary Tumor (Papilloma) Induction

The exposure periods before papillomas appeared on the mice with terminally detected metastases are listed in table 1. In relation to the averages for mice with topically induced tumors only, these data show an obvious lack of association between early papilloma appearance and subsequent involvement of distant sites. Apparently the principal factors determining susceptibility to skin-tumor induction are not identical with the factors regulating its metastatic spread.

Metastasis and Malignant Transformation of the Primary Tumors

The exposure durations until the induced tumors appeared grossly malignant for mice with metastases are listed in table 2. In comparison with the averages for mice with topical tumors only, the relative time at which malignant transformation was assumed to have taken place, and the relative duration of carcinogenic exposure for the individual members of an exposure group were not associated with subsequent metastasis from the primary malignancy.

Metastasis and Relative Life Span of the Tumor-Bearing Host

It is interesting to note in table 3 that metastasis to the lungs and lymph nodes did not shorten the life span of the tumor host in these experiments. It is also apparent that metastasis is not dependent alone upon continued life span of the host after initiation of the carcinogenic process.

Discussion

The similarities of both sexes in primary tumor genesis, *i.e.*, in the duration of their exposures before appearance of the initial papillomas, in the total duration of their exposures until comparable primary malignancies were induced, and in their experimental life spans as tumor hosts all suggest that the higher incidence in occurrence of metastasis among females is not dependent on a sex difference in local effect of the external carcinogens. The experimental carcinogenicity of pharmacologic doses of estrogen has been reported for a number of organs, *i.e.*, the

[9] The data show but one exception to the group pattern of predominance of metastasis among tumor-bearing females; *i.e.*, the DBA/2 mice exposed to the 20 percent solution of creosote. Of possible significance is the prolonged papilloma appearance time among the females of that group, ranging from 56 to 81 weeks, as compared with a 34- to 56-week range for the comparable exposed males. The limited gross size and histologic appearance of the females' primary tumors, terminally, suggest early stages of epidermoid carcinomas rather than advanced, flourishing growths with metastatic potentialities. Whether a somewhat decreased susceptibility to tumor development found in older mice is related to this observation is a possibility that cannot be determined from the data, in view of the small number of mice involved.

uterus, mammary glands, prostate, lymphoid tissue, kidney, and bladder (*9–11*); but the role of estrogen as a potentially endogenous topical carcinogen does not seem to be involved in this instance, for all tumors were induced with benzopyrene or creosote oil at a dose level capable of overshadowing hormonal effects. Furthermore, the principal sex-related difference under consideration does not involve the topically induced tumor directly. It appears more credible therefore, to inquire whether the higher incidence of metastasis in females may be related to an accelerating influence of estrogen on the invasive potentialities of a malignancy established by another agent. Although this possibility has not previously been demonstrated in relation to skin cancer, the accelerating effect of estrogen on the growth rate of methylcholanthrene-induced breast cancer in mice has been reported (*11*). In addition, clinical studies have demonstrated cases of primary and metastatic breast-cancer regression following mastectomy, ovariectomy, and adrenalectomy (*10–13*). Admittedly, primary breast cancer, unlike cancer of the skin, is believed to be influenced by endocrine secretions both in man and in the experimental animal. However, in view of the paucity of knowledge of physiologic factors determining metastasis, it is quite plausible although entirely speculative at this stage to suspect that physiologically "normal" levels of estrogenic hormone may account, in part, for the difference in incidence of metastasis reported herein.

Summary

1) Metastasis to the regional lymph nodes and lungs was observed as a fairly common finding in mice with induced primary epidermoid carcinomas of the interscapular and adjacent skin.

2) A sex difference in incidence of metastasis was observed, in which involvement of secondary sites among females exceeded that of the males. The difference suggests that the metastatic spread of epidermoid carcinoma may be related, at least in part, to endogenous female sex-hormone levels.

3) The duration of the carcinogenic exposure and dose of carcinogen, each a factor regulating tumor incidence and induction time, were not directly significant as factors regulating metastasis after induction of locally invasive carcinomas.

4) The relative susceptibility of individual animals to the induction of epidermoid carcinoma of the skin was found to have no obvious relationship to the occurrence of metastasis.

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Cytochemical Studies of Hyperplastic Alveolar Nodules in the Mammary Gland of the C3H/He CRGL Mouse [1, 2]

MARY NISHI HARKNESS, HOWARD A. BERN, MAX ALFERT, *and* NORMA O. GOLDSTEIN, *Department of Zoology and its Cancer Research Genetics Laboratory, University of California, Berkeley, California*

The significance of the hyperplastic alveolar nodule in the genesis of mammary cancer in the C3H mouse is under intensive investigation in our laboratory. Earlier studies (*1–3* and others) indicate that these structures are to be considered as preneoplastic lesions and that their occurrence correlates with tumor incidence in various strains of mice. In a recent study (*4*), we pointed out that hyperplastic alveolar nodules show a P^{32} uptake intermediate between that of normal inactive mammary gland and mammary tumor and approximately equal to that of normal prelactating tissue from mice on the 14th to 18th day of pregnancy. The present study was conducted to determine the existence of histologic and cytochemical differences between hyperplastic alveolar nodules and normal prelactating tissue. In addition, mammary adenocarcinoma was compared with nodules from the same mice.

Materials and Methods

Multiparous female mice of the C3H/He CRGL strain, about 8 months of age, provided the tissues used in this study. Hyperplastic nodules and small pieces of tumor were dissected out of the mammary glands of nonpregnant mice immediately after sacrifice. The mice were postlactational and had been isolated for at least 2 weeks prior to sacrifice. The nodules are recognizable in unfixed mammary tissue owing to their slight orange coloration, presumably related to the presence of iron-containing hemosiderin pigment (*5–7*). Normal prelactational tissue was obtained from mice on about the 15th day of pregnancy. These tissues were variously fixed and subjected to a series of cytochemical tests: Carnoy's fluid for the Feulgen reaction for deoxyribonucleic acid (DNA) (*8*), the azure-B method for nucleic acids (*9*), and the bromphenol-blue method for protein (*10*); 1 percent trichloroacetic acid in 80 percent ethanol for the demonstration of protein-bound sulfhydryl (SH) groups

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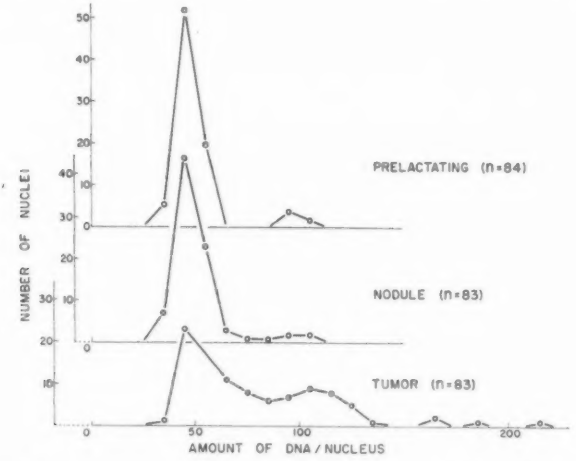
[2] This investigation has been supported by grants from the American Cancer Society, Inc. (MOR 27) and its California Division, and by Cancer Research Funds of the University of California. We are indebted to Professor K. B. DeOme for his indispensable assistance in the selection of hyperplastic nodules from fresh material for these studies.

(*11*, *12*); cold 80 percent ethanol for the Gomori methods (*13*) for alkaline phosphatase, incubating for 2 hours in sodium glycerophosphate or 15 minutes in α-naphthyl phosphate; 10 percent neutral formalin for straining of lipides in frozen sections with Sudan III and IV; Bouin's fluid for the periodic acid-Schiff (PAS) reaction (*14*) and for routine staining with hematoxylin and eosin; calcium-formol for demonstration of phospholipides by Baker's acid-hematein method (*15*, *16*), employing pyridine extraction of tissues fixed in weak Bouin's fluid as a control. Each of the qualitative cytochemical tests was generally performed on at least 5 pieces of prelactating and of tumorous tissues taken from 3 or more mice. Twelve to 18 nodules from 6 or more mice were similarly tested by each of the methods employed, except for the bromphenol-blue method, wherein only 2 to 3 samples of each tissue were examined.

Microspectrophotometric measurements of DNA were performed on Feulgen-stained sections to determine the chromosomal constitution of cells from the several mammary tissues (*17*). The cytophotometric method was employed as previously described (*18*). Prelactating tissue was obtained from 3 pregnant mice, and nodules and tumor tissue were obtained from 3 nonpregnant mice for these determinations. Between 20 and 30 measurements were made on each tissue sample, and frequency-distribution curves of DNA values were plotted (text-fig. 1). Whenever possible, sections of the 3 mammary tissues studied were affixed to the same slide for simultaneous treatment with each cytochemical procedure.



TEXT-FIGURE 1.—Frequency distributions of nuclear DNA content (Feulgen) in C3H mouse mammary tissues. *Note* correspondence of prelactating and nodule distributions; n=total number of nuclei measured.

Results and Discussion

A variety of structures exist which can be considered as hyperplastic nodules. These include both alveolar and ductal structures. The great

majority of nodules randomly selected from our C3H strain of mice, either in the unfixed state or from formalin-fixed, hematoxylin-stained mammary spreads, show a histologic picture resembling that in figure 2. Thus, "typical" or "average" nodules consist of lobules of alveoli in a prelactating state in glands that are otherwise in the resting state. We have deliberately selected these typical hyperplastic alveolar nodules for cytochemical studies in order to allow comparison with an appropriate control tissue: normal prelactating tissue from a pregnancy-activated mammary gland (fig. 1). In our current study we eliminated the few nodules that, after sectioning, revealed only limited secretory activity.

DNA Measurements

The distributions of DNA values from prelactating tissue and from hyperplastic nodules are practically identical. The nuclei are predominantly diploid; only a few preprophasic tetraploid and intermediate DNA values occur. The DNA distribution in tumor cells differs from the foregoing in two ways: 1) Correlated with the relatively higher mitotic activity that is evident in these tumors, the proportion of preprophasic tetraploid values and of intermediate DNA values, indicative of premitotic DNA synthesis, is greatly increased. 2) In addition, there are also DNA values greater than the tetraploid range, showing that polyploid cells occur in the tumors. These latter cells are completely absent from prelactating tissue and from nodules, and their presence differentiates the tumor from both non-neoplastic tissues. The DNA values observed in these tissues are in agreement with data obtained on various rodent tissues and tumors by Bader (*19*).

General Cytochemistry

In general, the several cytochemical techniques used reveal no differences between the prelactating and the hyperplastic tissues. Tumorous tissue, on the other hand, possesses some distinctive characteristics.

The PAS reaction revealed no diastase-removable material (glycogen) in any of the mammary tissues (*20*). In general, the PAS picture in nontumorous tissues corresponds roughly to that seen after use of the method for alkaline phosphatase. Luminal borders and secretory masses are intensely reactive, as is the basement membrane region (figs. 3 and 4). The myoepithelium itself appears largely PAS-negative. In the tumor, not only is the luminal border clearly defined by its reactivity but also the basement membrane region, and the results contrast with those of the alkaline-phosphatase method (*cf.* figs. 5 and 8).

The alkaline-phosphatase method has become recognized as a valuable adjunct for the visualization of myoepithelium in a variety of glands: mammary (*20–25*), salivary (*24, 26*) and ceruminous (*27*), and the myoepithelial ("basket") cells of the mouse mammary gland are clearly evident in association with both normal and hyperplastic alveoli (figs. 6 and 7). We are in agreement with Richardson and Pearson (*23*) as to

the presence of alkaline phosphatase in the myoepithelium of hyperplastic nodules, as well as at the luminal borders, in capillary endothelium, and in intracellular (largely apical granules) and luminal secretory material. The luminal outlines are clearly indicated in the mammary adenocarcinomas showing acinar patterns (fig. 4 in *28*), but the other sites of activity seen in non-neoplastic tissues are generally absent from the tumors studied by us (fig. 8). In fact, apical alkaline-phosphatase activity was considerably more intense in the tumors examined than in the nodules or prelactating tissues.

Both the alkaline-phosphatase method and the PAS reaction readily establish the essentially acinar pattern of many C3H mammary carcinomas. This pattern can also be elucidated by visualization of the apically located Golgi apparatus, as was pointed out by Dalton (*29*).

The hyperplastic alveolar nodules in late pregnancy seem to become filled with milk prematurely, unlike the adenomatous nodules described by Fekete (*30*) in DBA mice, and are readily identifiable in the unfixed mammary gland. These areas generally react, as does any area of normal lactating gland, to the local application of oxytocin (accomplished by semimicroinjection of the stimulant into the region of the nodule). This observation (Bern and Crosby, unpublished) verifies the essential normality of the myoepithelium in the typical hyperplastic alveolar nodule.

By ordinary histologic methods, the alveolar epithelium of the prelactating tissue and that of the typical hyperplastic nodule are indistinguishable (figs. 1 and 2). Most cells of both tissues contain large vacuoles, mostly apical, and the secretion mass in the lumen also has a vacuolated appearance. The sudanophilia of the vacuolar contents and of the secretion mass is apparent when formalin-fixed frozen sections are suitably stained. The studies of Johnson and Dutch (*31*) established the existence of considerable neutral fat in "physiologically stimulated" (late pregnant) mammary tissue, as well as in normal resting tissue of strain DBA mice. In their studies, it would seem impossible to eliminate the major contribution of the adipose tissue in resting mammary glands to total fat determinations. Mammary tumor, on the other hand, possessed only a minute amount of neutral fat, and sudanophilic material is virtually absent from tumor in our cytochemical preparations.

Baker's method for presumed phospholipide reveals pyridine-extractable material similarly distributed in prelactating and hyperplastic tissues. Fine cytoplasmic granulation is evident, especially apically. Where large apical secretion vacuoles are present, the peripheries of these vacuoles are generally strongly reactive. Occasional large, dense globules are also present in the cytoplasm and in the lumen. In some tumors, some lightly staining granules are present throughout the cytoplasm without any evidence of apical orientation when an acinar pattern is otherwise evident.

Azure-B-treated tissue sections demonstrate a pronounced basophilia in the finely granular cytoplasm and in the nucleoli of all mammary tissues. The nucleoli of the nodule parenchymal cells appear more baso-

philic than those of prelactating and tumor cells, but this difference was not quantified. The bromphenol-blue method shows a proteinaceous secretion in both prelactating and hyperplastic alveoli. Despite the presence of an acinar pattern in the tumors studied, the bromphenol-blue method revealed no reactive secretory material in the demonstrable lumina. Protein-bound SH groups are distributed throughout the cytoplasm of the secretory cells in both normal prelactating and hyperplastic tissues. The reaction is particularly evident around the larger intracellular vacuoles and at the apical borders. Neoplastic tissue appears more intensely reactive for SH than non-neoplastic. Where an acinar structure is present in the tumor, the lumen is clearly outlined by the apical concentrations of SH groups.

Partially relevant to our findings are the observations of Rawlinson (*5*, *6*) who found that stainable iron deposits occur in hyperplastic nodules of DBA mice, as well as in the normal glandular tree, although reduced in amount in pregnancy (*5*). Such deposits are absent from frankly neoplastic tissue (*6*).

The general histologic and cytochemical picture reveals no significant differences between normal prelactating mammary tissue and hyperplastic alveolar nodules. The nodules show no morphologic criterion characteristic of neoplastic tissue, which is not also evident in prelactating tissue. Some workers (*e.g.*, *2*) find characteristic stromal differences between nodules and normal lobules, with more connective tissue associated with the former. However, in our experience this is neither a consistent nor a reliable criterion and is not supported by our current study. Differences certainly exist between nodules and normal prelactating tissue in terms of growth potential and hormone sensitivity, but none is apparent at the cytochemical level, nor at the level of metabolic activity as measured by P^{32} uptake (*4*). Preliminary observations indicate that differences may exist at the ultramicroscopic level, however, and these will be reported separately (*32*).

Summary

Normal prelactating tissue and hyperplastic alveolar nodules from mammary glands of C3H/He CRGL mice have been investigated by a series of cytochemical methods and compared with mammary adenocarcinoma. Cytophotometric determinations of nuclear deoxyribonucleic acid were also made on these tissues. The adenocarcinoma was clearly distinguished from the non-neoplastic tissues in a variety of ways, including absence of alkaline phosphatase-positive myoepithelium; intense apical alkaline-phosphatase activity; absence of lipide-containing vacuoles and of notable phospholipide accumulations; and presence of an appreciable proportion of preprophasic tetraploid and higher polyploid DNA values. On the other hand, no consistent difference on the morphologic and cytochemical level was found to distinguish between the normal prelactating tissue and the typical hyperplastic alveolar nodule, which are thus considered as representing essentially similar tissues present in mammary glands under different physiologic conditions.

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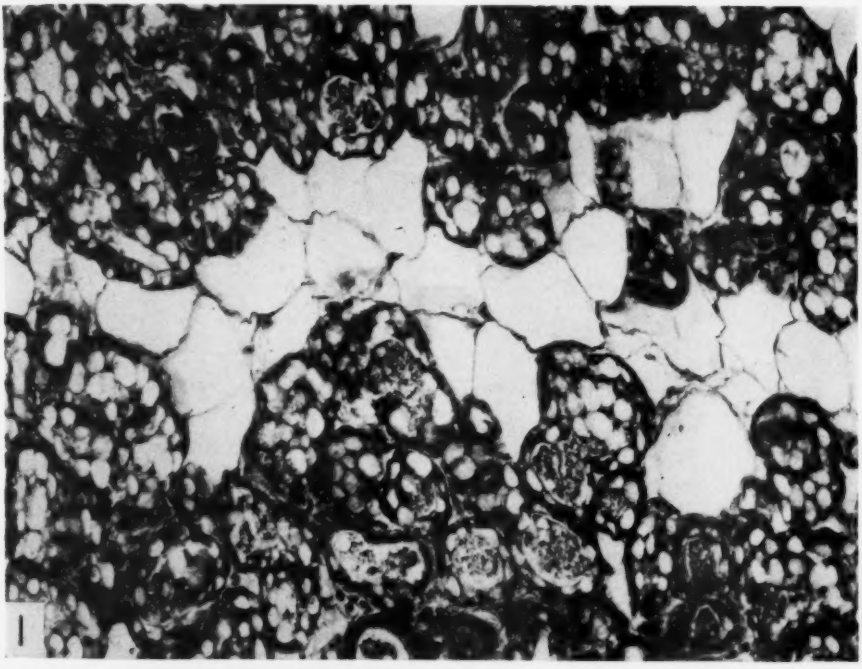
PLATE 87

Mammary tissues stained with hematoxylin and eosin. × 300

FIGURE 1.—*Prelactating tissue* from C3H mouse, approximately 15 days pregnant.

FIGURE 2.—*Hyperplastic alveolar nodule* from multiparous, tumor-bearing C3H mouse.

Photomicrography by Mr. Victor Duran



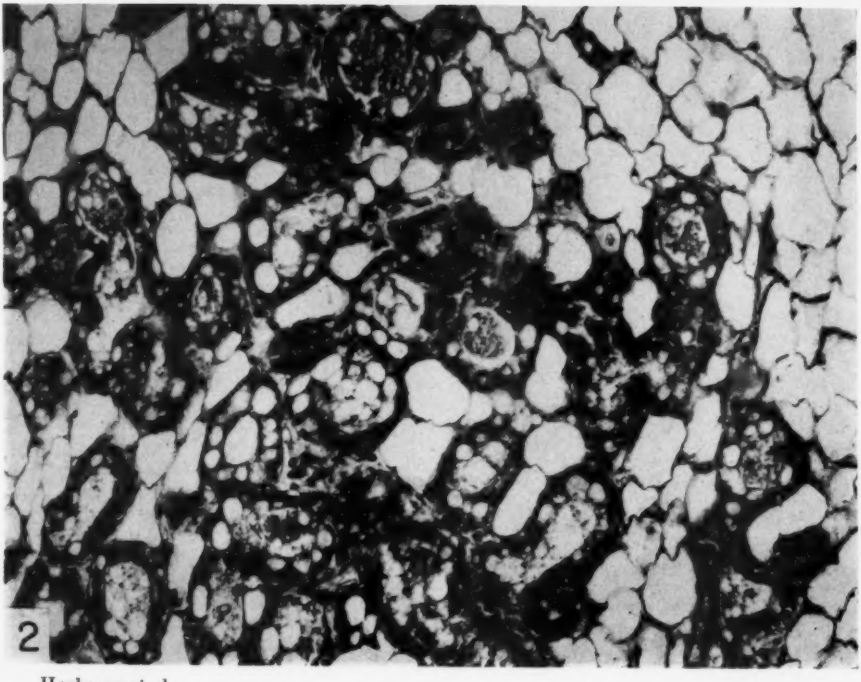


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PLATE 88

Bouin's fixation; PAS reaction. × 300

FIGURE 3.—*Prelactating tissue. Note* intensely PAS-positive secretion and basement membrane region.

FIGURE 4.—*Hyperplastic nodule. Note* as above.

FIGURE 5.—*Mammary adenocarcinoma. Note* outlining of acinar lumina by PAS-positive cell apices; PAS-positive basement membrane region.

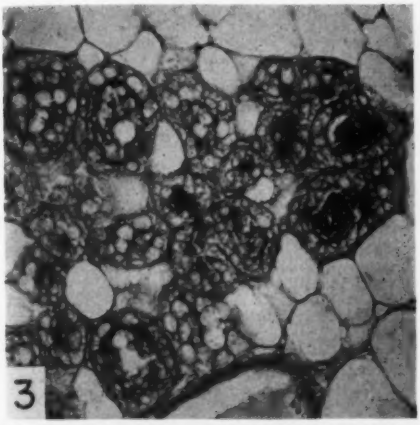
Cold 80 percent ethanol fixation; Gomori's method for alkaline phosphatase; incubated in sodium glycerophosphate for 2 hours. × 300

FIGURE 6.—*Prelactating tissue. Note* intense alkaline-phosphatase activity in myoepithelium; moderately reactive luminal secretion, cytoplasmic granules, and apical cell borders.

FIGURE 7.—*Hyperplastic nodule. Note* as above.

FIGURE 8.—*Mammary adenocarcinoma. Note* intensely reactive cell apices outlining acinar lumina; absence of myoepithelium.

Photomicrographs by Victor Duran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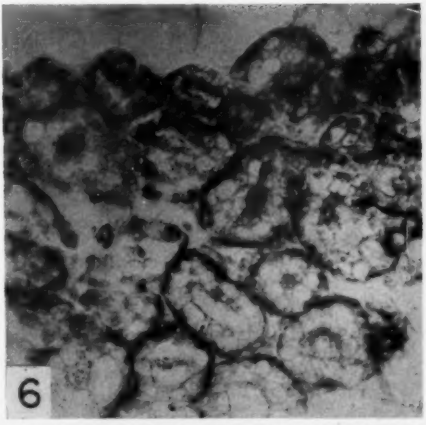
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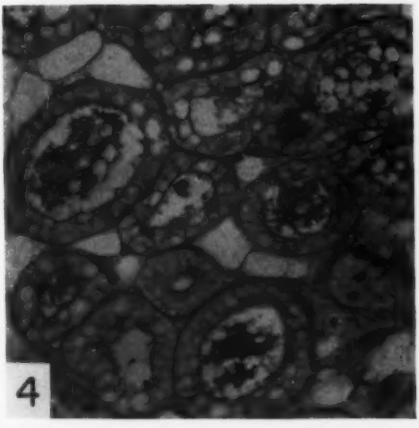
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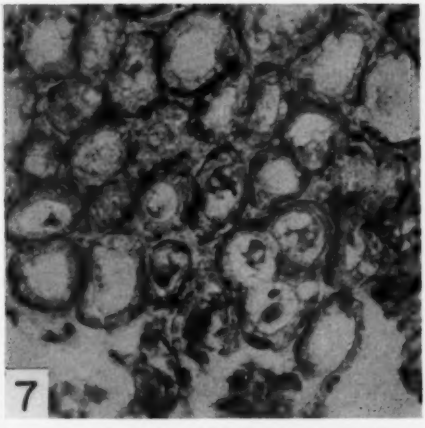
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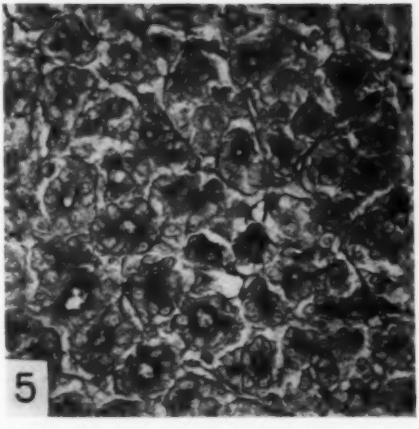
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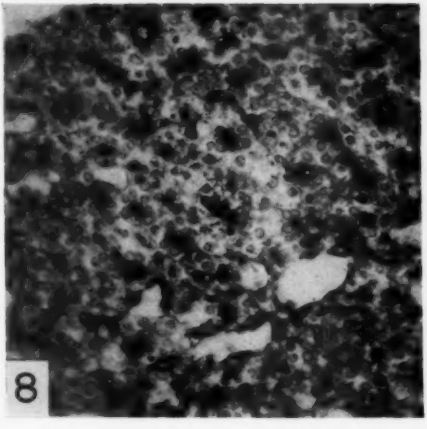
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Bilateral Mammary Cancer in Italian Women [1]

PIERO MUSTACCHI,[2] ANNA PANDOLFI,[3] *and* PIETRO BUCALOSSI,[4] *Biometry Branch, National Cancer Institute,*[5] *Bethesda, Maryland, and Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy*

This note presents the results of an actuarial analysis of the occurrence of a second independent mammary cancer in a group of 3,818 women with cancer of the breast. The simultaneous or successive appearance of these bilateral neoplasms has been analyzed separately. In addition, we have calculated age-specific incidence rates for a second mammary cancer on the basis of the experience of this group of hospital patients.

To our knowledge, the literature on bilateral breast cancer includes only one other article (*1*) where the group under study (941 women) was the object of an actuarial analysis. The data presented there supported the conclusion that, in those women, the remaining breast was likely to develop cancer more frequently than the breast of otherwise healthy persons. However, those data provided no information regarding simultaneously occurring bilateral cancers, an eventuality which in larger series is far from being a rarity (*2, 3*).

Criteria

We believe that the demonstration of *in situ* cancer within the duct system of the contralateral breast affords the soundest criterion for establishing beyond reasonable doubt the independence of 2 breast cancers. However, useful as it is in individual cases, this norm fails to take into account the possibility that 2 breast cancers might still be independent in the absence of this specific microscopic demonstration. Variations in the general histologic pattern of 2 breast cancers will lend contributory support to the clinical impression that they are independent, but it is generally conceded that such microscopic differences might still represent histologic variants of the same metastasizing neoplasm. It has also become part of the present concept of independence of 2 breast cancers, that no

[1] Received for publication May 20, 1957.

[2] *Present address:* Departments of Medicine and Preventive Medicine (Epidemiology), University of California of Medicine, Parnassus at Third Ave., San Francisco 22, Calif.

[3] Assistant, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy.

[4] Chief, Medico-Surgical Division and Director, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy.

[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

metastases from the first (treated) neoplasm should be clinically present or suspected in order to consider the new cancer an independent primary. These restrictions, safe as they are, will exclude from the definition of "independent primary" an unknown number of cases in which a second independent neoplasm develops in the presence of metastases of the first one. Admittedly, in the presence of metastatic breast cancer, a malignant mass in the remaining breast is likely to be just another metastasis. Still we have no reason as yet to believe that the presence of a metastasizing mammary cancer will immunize its host against a new primary cancer of the breast. This is, however, the assumption implicit to these restrictive criteria. As a corollary to these criteria, a certain period of freedom from recurring or metastasizing disease has been expected to elapse between first and second cancers. Originally placed to preferably more than 2 years by Guiss (*4*), and shortened to 1 year by Farrow (*3*), this time limit was interpreted as affording a safety margin after which a second breast cancer could be considered an independent primary. We believe that setting any arbitrary time limit raises valid questions. In fact, available information indicates that the mortality of women with localized breast cancer continues to be greater than that of the general population even 10 years after diagnosis and treatment (*5*). This would suggest that clinically silent cancer may become reactivated even after several years of quiescence; in which event even a 2-year limit may not contribute substantially to the firmness of a diagnosis of independent primary cancers.

In our study the decision as to which cases were to be considered double primaries, was based on the clinical judgment passed by the surgeon at the time the patients were examined and operated. We believe that a surgeon who plans to treat radically cancer of the remaining breast does so because he is confronted with a composite clinical picture which convinces him that this new growth cannot be suspected in any way to derive from extension or metastasis of the earlier cancer. In other words, we have accepted his judgment because all the clinical evidence available to him at that moment supported his impression that he was dealing with a new primary. Under these circumstances, the lapse of time between the original mastectomy and the diagnosis of the second primary was of little moment. Actually, one would find little excuse for any surgeon who, even though convinced that a radical procedure could be curative, would refrain from performing it just because the second primary neoplasm happened to be diagnosed before a preset time limit had elapsed.

The criteria used here are similar to those suggested by Guiss (*4*), except for the time limit placed on the diagnosis of the second cancer. We realize, as was mentioned before, that in women with metastatic disease an unknown number of genuine second primaries may have gone unrecognized and, consequently, would have been excluded from the study. We do not know how this difficulty could have been solved, nor how we could have avoided excluding an unknown number of cases of independent double primary breast cancer that may have presented general medical contraindications to a second radical mastectomy.

Material

During the 28-year period ending December 31, 1955, breast cancer was diagnosed in 3,818 women attending the Istituto Nazionale per lo Studio e la Cura dei Tumori. A second independent breast cancer was diagnosed in 109 instances: 26 times (0.7% of 3,818) simultaneously with and 83 times (2.2%) successively to the contralateral cancer.[6]

Method

Analysis of Successively Occurring Bilateral Breast Cancer

The 3,792 patients (3,818 minus 26 with simultaneous breast cancer) were distributed by age at diagnosis of their first neoplasm. By definition, their risk of developing a second cancer was considered to begin at that time. For 86 women, this period terminated when the risk materialized in the form of a second independent primary breast cancer. For the other ones, the period of life at risk of a second cancer terminated either at the time they died or were last seen free of metastatic or recurrent disease. In the absence of age-specific incidence[7] rates for breast cancer in Italian women, we have used those published for white women, all regions, in the survey on morbidity from cancer conducted in 1947–48 by the National Cancer Institute in 10 metropolitan areas of the United States (*6*). These rates were applied to the person-years at risk contributed to this study by the Italian group of women. "Expected" values were thus obtained and were contrasted with the observed figures.

Analysis of Simultaneously Occurring Bilateral Breast Cancer

At the time of diagnosis of cancer in 1 breast, 26 of the 3,818 women had an independent cancer in the opposite side. We have elected to compare this figure with what would have been the prevalence[7] of cancer in a group of white women, with similar age distribution, residing in the metropolitan areas of the United States. Prevalence rates, calculated from the data published in the report on morbidity from cancer (*6*), were applied to the distribution by age at diagnosis of cancer of the Italian group of patients to yield "expected" values.

Calculation of Age-Specific Incidence Rates for a Second Independent Breast Cancer in Women with Cancer of the Breast

The number of the second primaries observed during each age interval was divided by the number of person-years at risk contributed by these

[6] Carroll and Shields, reviewing 17,048 operated cases collected from various sources, found that 1.4 percent had simultaneous bilateral lesions and 3.3 percent developed bilateral nonsimultaneous cancers (*2*). Farrow reported that of 4,908 primarily operable cases 0.4 percent had synchronous bilateral lesions and 3.2 percent successive bilateral breast cancer (*3*).

[7] Incidence indicates the number of *new* cases occurring in a defined population within a given period of time. Prevalence indicates the number of cases known to exist within a defined population at any particular time. For this reason, in the calculation of "expected" values, age-specific incidence rates were applied to the number of person-years at risk, while age-specific prevalence rates would be applied to the number of people present at a given time.

patients to each corresponding age period. This yielded average annual age-specific incidence rates (table 1).

Results and Comments

On the basis of the United States incidence data for primary breast cancer in white urban women and the period during which the Italian patients had been observed, one would have expected only 13 new successive cancers of the breast. The Italian group had 83 such independent mammary cancers. This is a definite overrepresentation (table 1). We believe that its significance is enhanced by the realization that the incidence rates described in the report on "Morbidity from Cancer in the

TABLE 1.—*Observed and "expected"* numbers of consecutive second primary breast cancers in a group of 3,792 women with breast cancer. Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy, 1928–55.*

Age group	Number of patients, by age, at diagnosis of 1st cancer	Number of person-years at risk	Observed number of 2nd cancer, by age, at diagnosis	"Expected"* number of breast cancers	Observed average annual incidence rate per 100,000
All ages	3, 792	8, 213	83	13. 34	—
15–19	1	0	0	0. 00	—
20–24	3	2	0	0. 00	—
25–29	42	38	0	0. 00	—
30–34	144	181	0	0. 05	—
35–39	365	540	6	0. 32	1, 110
40–44	597	1, 114	13	1. 09	1, 167
45–49	690	1, 579	18	2. 19	1, 140
50–54	547	1, 533	11	2. 50	718
55–59	482	1, 185	10	2. 16	844
60–64	381	915	11	2. 06	1, 203
65–69	312	663	6	1. 61	905
70–74	150	327	3	0. 87	916
75–79	67	119	5	0. 42	4, 202
80–84	11	16	0	0. 06	—
85–89	0	1	0	0. 00	—

*"Expected" according to the age-specific incidence rates for breast cancer among white women of 10 United States metropolitan areas (6). Numbers rounded to the nearest hundredth.

United States" are the highest reported in the literature. Again, although this impression cannot be substantiated, it seems possible that, in general, Italian women might have experienced a greater number of pregnancies than American women living in the surveyed areas. If this were so, and if the negative association believed to exist between parity status and breast cancer were present also in Italy, then additional circumstantial support would be offered to the validity of the difference between observed and "expected" values. Also, although mortality rates are at best of limited value as an indirect index of incidence, it may be worth mentioning here that the Italian mortality rate from cancer of the breast falls considerably short of the one experienced in the United States (7).

Table 2 indicates that on the basis of the United States prevalence figures, 9 cancers would have been "expected" against the 26 observed. Here too, there are certain considerations that may add significance to these differences. First, it is admittedly impossible to enumerate completely cases diagnosed in an earlier year that are not under active observation and treatment at the time of the survey. Still, if one takes the rather extreme position that one third of the breast cases were missing from the prevalence survey, this would raise the expected number from 9 to 12, which remains significantly short of the 26 observed. Next, the United States prevalence figures include the cases known to be alive at any time during the survey year, while our 26 cases represent a prevalence limited, so to speak, to the time of the diagnosis of the contralateral

TABLE 2.—*Observed and "expected"* numbers of simultaneous bilateral breast cancers in a group of 3,818 women with cancer of the breast Instituto Nazionale per la Sludio e la cura dei Tumori, Milano, Italy, 1928–55.*

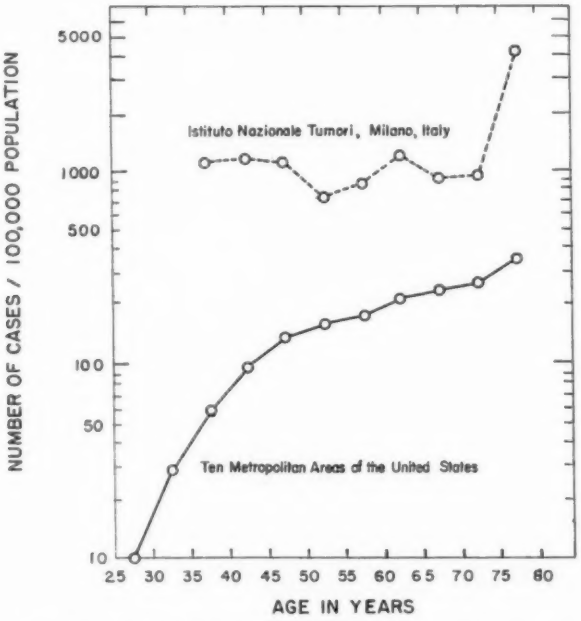
Age group	Distribution of patients, by age, at diagnosis	Observed number	"Expected" number*
All ages	3, 818	26	9. 26
15–19	1	0	0. 00
20–24	3	0	0. 00
25–29	43	1	0. 01
30–34	146	2	0. 06
35–39	368	3	0. 33
40–44	600	3	0. 93
45–49	700	10	1. 53
50–54	548	1	1. 43
55–59	483	1	1. 41
60–64	383	2	1. 35
65–69	314	2	1. 19
70–74	151	1	0. 64
75–79	67	0	0. 33
80–84	11	0	0. 05

*"Expected" according to age-specific prevalence rates for cancer of the breast among white women of 10 United States metropolitan areas (*6*). Numbers rounded to the nearest hundredth.

neoplasm. Finally—and this might be argued also for the previous part of the study—the question arises as to whether a woman with only 1 breast is not in effect exposed only to half of the risk of developing breast cancer. We do not know of any way whereby, with the information presently available, this question could be satisfactorily answered. All we can say is that if this suggestion were a fact, it would result in an even greater discrepancy between observed and "expected" figures; this would occur for the "expected" values calculated on the basis of both incidence and prevalence data.

The figures presented in the last column of table 1 paraphrase the previous findings. However, an additional facet gains prominence when the age-specific incidence rates for the second primary breast cancer observed in Milan is contrasted with the incidence rates for breast cancer

of the white American women surveyed during the cancer morbidity study of 1947–48 (*6*) (text-fig. 1). While these observed rates reflect a significant increase over the basic U. S. risk, they also indicate that, once it manifests itself, the risk of developing a second primary breast cancer apparently does not vary with age. Table 3,[8] where we have considered only broad age groups in order to obtain numbers large enough to yield meaningful ratios, further documents this point. For all ages combined, the risk for a second primary mammary carcinoma to develop in our group of Milanese women was 6.2 times greater than the basic U. S. risk for a first breast cancer. This increase ranged from about 5-fold for women over the age of 55 to 13-fold for women under age 45. Because the breast-cancer incidence rates for the United States urban white females increase with age, the ratios shown in table 3 tend to decline with advancing age.



TEXT-FIGURE 1.—Age-specific incidence rates for primary breast cancer, white females, 10 metropolitan areas of the United States, 1947–48; and for a second independent breast cancer, Milano, 1928–55.

This may seem to be at variance with what has been reported for the incidence of multiple cancers by Watson (*8*). However, this author, in reporting the curve for the incidence of a subsequent independent malignant tumor among patients with cancer, has purposely excluded from his calculations those occurring in the same organ or system. Consequently, our observations, based on independent primaries occurring in a paired organ,

[8] Based on table 1.

TABLE 3.—*Ratio of observed to "expected"* numbers of consecutive second primary breast cancers in a group of 3,792 women with breast cancer—by broad age groups. Instituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy, 1928–55.*

Age group	Observed number of 2nd primary breast cancer, by age at diagnosis *A*	"Expected"* number of breast cancers *B*	Ratio $\frac{A}{B}$ *C*
All ages	83	13. 34	6. 2
Under 45	19	1. 46	13. 0
45–54	29	4. 69	6. 2
55–64	21	4. 22	5. 0
65 and over	14	2. 96	4. 7

*"Expected" according to the age-specific incidence rates for breast cancer among white women of 10 United States metropolitan areas (*9*). Numbers rounded to the nearest hundredth.

represent a special situation not included in his study. In view of the findings of Macklin (*9*), Woolf (*10*), and others with regard to the inheritance of breast cancer, our results were to be expected. This group of Milanese women had the genetic background of susceptibility to mammary carcinoma and only the necessary environmental stimulation was needed to initiate the second primary breast cancer.

Summary

1) Among 3,818 Italian women with breast cancer seen at the Istituto Nazionale per lo Studio e la Cura dei Tumori, 109 developed a second primary cancer in the opposite breast.

2) Of these 109 second primary cancers, 83 were diagnosed successively to the contralateral 1, against 13 expected; and 26 were diagnosed simultaneously with the original cancer, while only 9 would have been expected.

3) For all ages combined, the remaining breast of the Italian women already operated for a contralateral mammary carcinoma appeared to be attacked by cancer 6.2 times more frequently than the breast tissue of otherwise "healthy" women living in the metropolitan areas of the United States. This increase ranged from about 5-fold for women over the age of 65 to 13-fold for women under age 45.

4) The validity of the conclusion that there is a marked overrepresentation of observed bilateral breast cancer rests on the assumption that it was legitimate to apply United States incidence and prevalence rates to the Italian group under study.

5) Our observations would indicate that the incidence rates for a second primary breast cancer are not substantially influenced by advancing age.

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Separation of Actinomycin C Fractions and Experimental Evaluation of their Carcinolytic and other Biological Properties [1]

J. De Cort, *Dr. Med.*, *and* L. Delcambe, *Dr. Sc.*,[2]
Institut Vésale, Medical Faculty, University of Louvain, Belgium, and Centre de Recherches pour la Pénicilline et les autres Antibiotiques, Liège, Belgium

In 1940, actinomycin A was isolated by Waksman and Woodruff (*1,2*) from a new actinomycete—*Streptomyces antibioticus*. Actinomycin A exerted a marked cytotoxic activity especially selective for the spleen and the lymphatic system, but because of the high toxicity (*3, 4*) of this product further investigations were not made. Later, a number of similar substances were reported: actinomycin B isolated by Lehr and Berger (*5*) and further studied by Dalgliesh *et al.* (*6, 7*); actinomycin C, X, and I by Brockmann *et al.* (*8–17*); actinomycin J by Umezawa *et al.* (*18, 19*); actinomycin D by Manker *et al.* (*20*). Carcinostatic effects of actinomycin C on sarcoma 37, the Ehrlich acites tumor in mice, and on the Walker carcinosarcoma 256 of the rat were observed by Hackmann (*21–23*). Tumor-cell suspensions were mixed with the antibiotic solutions and implanted. A remarkable inhibition of takes was noted. The Walker tumor was also treated *in vivo* with some success. Field *et al.* studied the activity of actinomycin C on Crocker sarcoma 180, RC adenocarcinoma, and mouse leukemia L4946 (*24*). Inhibition of tumor growth of about 50 percent was observed, but only in the groups receiving a high dosage which caused a mortality of about 50 percent. Encouraging results of actinomycin C therapy were first described by German authors, especially in Hodgkin's disease (*25, 26*).

Brockmann (*8–16*) has shown that these antibiotics consist of a mixture of different components. This is true in the actinomycins we have studied. The present work was done in an attempt to ascertain which components of these mixtures were most active as tumor inhibitors.

Experimental

Preparation and Separation

In a preceding publication (*27*), one of us described in detail the preparation, on a semi-industrial scale, of submerged cultures of actinomycin produced by cultures of *Streptomyces* strain 67 isolated by Welsch (*28–30*).

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We reported the antibacterial activity, as compared with the toxicity for mice and for *Artemia salina*, of the fractions separated by adsorption chromatography on alumina (*31*).

Various strains of *Streptomyces* were studied: strains 17, 32, 36, 51, 67, and 95 from Welsch; strains 218 and 616 from De Somer; and one strain from Giolitti.

Paper chromatography was performed according to Brockmann's technique (*8–15*). This technique showed that all the strains of *Streptomyces* examined produced 6 identical fractions, but in different proportions. The fractions were designated C1, C2, C3, Co, Coa, and Cob because these substances seemed to be identical with those described by Brockmann as actinomycin C (Sanamycin,® Bayer). Both contain D-alloisoleucine, a typical amino acid of actinomycin C. The major components (C1, C2, C3) of these complexes occupy the same relative positions on circular comparative paper chromatograms with different systems of solvents. The strains 17, 32, 36, and 95 produce small amounts of C1; strains 51, 67, and 616, much more, while strain 218 and Giolitti's strain give large amounts. Paper chromatography allowed us to control easily the purity of the isolated fractions.

It is possible to separate the 6 constituents on a cellulose column with the solvents indicated by Brockmann (*13*, *14*). We easily obtained a separation by first isolating 2 groups of substances by adsorption chromatography on alumina in benzene with ethyl acetate as an eluent. The first group (C1, C2, and C3) is then fractionated on cellulose powder with the solvent pairs *n*-butyl ether, butanol, and sodium cresotinate 10 percent (5 : 1 : 5). These fractions have been *crystallized* from ethyl acetate. For the second group (Co, Coa, Cob), *n*-butyl ether, butanol, sodium cresotinate 10 percent, and ammonia (5 : 3 : 4 : 0.1) are used.

The adsorption spectrum showed maxima at 240 and 440 mμ. These maxima are influenced by exposure of the products to daylight or ultraviolet rays. Therefore, fractions should be separated and conserved in the dark.

For the determination of amino acids, the fractions were hydrolyzed with 6 *N* HCl during 48 hours at 100° C. in sealed glass tubes. After cooling, the acid was expelled completely by vacuum distillation. The residue, dissolved in water, was analyzed by paper chromatography with the solvent pairs: butanol, acetic acid, water (4 : 1 : 5). After 4 to 5 hours' migration, the paper was dried and sprayed with ninhydrin reagent. The crude product and the fractions contained the following amino acids: threonine, sarcosine (*N*-methylglycine), proline, valine, *N*-methylvaline, and alloisoleucine. Fraction C1 contains large amounts of valine, while fraction C3 has none. Fractions Co and Coa have a high content of alloisoleucine.

Toxicity and Antibiotic Activity

The activity has been tested on *Bacillus subtilis* by a broth-dilution technique. Fractions C1, C2, and C3 react similarly and have a higher degree of activity than Co, Coa, and Cob.

Toxicity tests on mice, performed according to the technique described in a previous communication (*31*), gave an LD50 between 0.9 and 1.8 mg. per kg. for C1, C2, and C3 and the unfractionated actinomycin. The other fractions are less toxic. The toxicity of the unfractionated actinomycin and for the first 3 pure fractions is about the same for *Artemia salina*, as indicated in table 1.

TABLE 1.—*Biological activity of actinomycins extracted from strain 616 cultures*

	Crude product	Fractions: C1	C2	C3	Co	Coa	Cob
Minimum concentration inhibiting *B. subtilis* (γ/ml.)	0.2	0.2	0.14	0.2	19	50	28
LD50 for mice (*mg./kg.*)	1.6	1.8	0.9	1.6	8	24	41
LD50 for *Artemia salina* (γ/ml.)	1.6	0.8	0.9	0.9	7	35	50

Comparative Effects on Experimental Tumors in Mice

In a previous report, the activity of actinomycins produced by the strains 616, 304, and 218 from De Somer and of actinomycin C (Sanamycin,® Bayer) on sarcoma 37 and on lymphosarcoma T. 24.179 was described (*32*). Five intraperitoneal injections, given every other day, seemed to be the optimal dose sufficient for obtaining a good inhibition of tumor growth.

Results

Effect on Carcinoma T. M. 8013

This tumor is a transplantable mammary carcinoma that grows rapidly in strain C57BL mice and their F_1 hybrids and kills the host in about 3 weeks. F_1 hybrid mice of both sexes, obtained by mating females of the C57BL strain with males of the CBA strain, both obtained from the Cancer Institute of Amsterdam, weighing 20 to 24 gm., were used for this experiment. The macroscopic, non-necrotic parts of 3 tumors of passage 179, taken 10 days after inoculation into the host, were homogenized and each animal was implanted subcutaneously with 0.1 ml. of tumor paste, *i.e.*, passage 180). Nine groups of 10 mice each, comprising 5 males and 5 females, were inoculated. Eight of these groups were treated, the ninth served as a control group. All animals were maintained in groups of 5 to a cage. They were maintained on a stock diet (Protector) and water *ad libitum*.

The pure fractions and the unfractionated actinomycin were dissolved in double-distilled sterile water and injected intraperitoneally every other day in a volume of 0.25 ml., beginning 24 hours after implantation. Previous experiments revealed that no important retardations of tumor growth could be obtained if treatment was started on far-advanced tumors, 1 or 2 weeks after implantation. Five injections on 9 days were administered.

Two weeks after implantation, when ulceration of the control tumors began, all animals were sacrificed. A complete autopsy was performed and the tumor, liver, and spleen were removed and weighed.

The results of this experiment are summarized in table 2. Actinomycin 616 (Louvain), $5 \times 10\gamma$, and actinomycin C (Bayer), $5 \times 2\gamma$, inhibited the growth of carcinoma T. M. 8013 almost completely. The fractions C1, C2, and C3 had a remarkable retarding effect on the growth of the tumor; the average tumor weights reached only 12, 27, and 42 percent of the controls. The fractions Co and Coa were less active, the resulting tumors weighing 65 and 88 percent.

TABLE 2.—*Mammary carcinoma T. M. 8013, passage 180 in (C57BL × CBA)F_1 mice*

Group	Dose (γ)	Avg. weight change of animals (*gm.*)	Avg. weight of tumors* (*centigram*)	Controls (*percent*)	Avg. weight of spleen* (*centigram*)	Controls (*percent*)
Controls	—	+2.3	162.7 ± 32.9	—	20.9 ± 1.0	—
Actinomycin C (Bayer)	5 × 2	−0.1	3.8 ± 1.2	3	11.6 ± 1.5	56
Actinomycin 616 (Louvain)	5 ×10	−2.3	4.3 ± 1.5	3	7.7 ± 1.1	36
Actinomycin 616 (Liège)	5 × 2	−0.7	29.4 ± 6.6	19	19.9 ± 3.1	95
C1	5 × 2	−3.7	18.6 ± 4.3	12	5.1 ± 0.9	24
C2	5 × 2	−0.2	42.3 ± 5.3	27	16.3 ± 0.6	78
C3	5 × 2	+1.3	65.0 ± 20.1	42	18.9 ± 2.3	90
Coa	5 × 2	+3.7	100.9 ± 29.7	65	29.8 ± 3.1	142
Co	5 × 2	−0.3	137.4 ± 29.7	88	26.6 ± 4.9	126

*±Standard Error of the mean.

The spleen of the treated animals weighed 24 percent of the controls in the C1 group, 78 percent in the C2 group, and 90 percent in the C3 group. The average weight of the spleen in the Co and Coa groups exceeded that of the controls (142 and 126%). The actinomycin 616 treated group had an average spleen weight of 36 percent and the actinomycin C treated group 56 percent.

Statistically, the difference between control and treated groups is significant ($P \leq 0.05$) for the weight of the tumors, except for the Co and Coa treated groups. C1, C2, and C3 are most active. The tumor-inhibiting activity of the pure fractions on carcinoma T. M. 8013 is not completely comparable with the toxicity of these products, for C1 has about the same LD50 as C3 but seems to be more active on T. M. 8013. No important weight changes of the liver have been found in most of the treated groups.

This experiment was repeated with passage 191 of the same tumor grafted in 57 males of the C57BL strain and F_1 hybrids obtained by mating WLL females with C57BL males, weighing between 19 and 22 gm. The control group consisted of 6 C57BL and 3 WLL × C57BL mice, while all the other experimental groups were composed of 3 C57BL and

TABLE 3.—*Mammary carcinoma T. M. 8013, passage 191 in C57BL and (WLL × C57BL) F_1 mice*

Group	Average weight change of animals (*gm.*)	
Controls	+5.3	
	Dose: 5 × 2γ	*Dose: 5 × 1γ*
Actinomycin 616	−1	+4.4
C1	+2.5	+2.2
C2	+1	+4.9
C3	+3.6	+5.1

3 WLL × C57BL mice. The same technique as in the preceding experiment was used. Treatment was started 24 hours after implantation, but each time 2 groups were treated with the same substance. The first group with the same dose, as previously used: 5 × 2γ; the second group with a lower dose of 5 × 1γ.

Because of the different growth rates in the C57BL and in the hybrids, results were calculated separately. Tumor T. M. 8013 grows faster on C57BL mice, who die about 3 weeks after implantation, than on WLL × C57BL hybrids who may survive more than 1 month under the same experimental conditions. Thus, the influence of the growth rate of the tumor on the sensitivity of the actinomycin treatment could be studied (table 3).

Even with these small experimental groups the results obtained with doses of 5 × 2γ are similar to those of the preceding experiment especially in the C57BL groups. Tumor weights averaged 8.9 percent of the controls in the actinomycin 616 treated group and, respectively, 14.2, 13.0, and 67.6 percent in the groups treated with the fractions C1, C2, and C3, as shown in table 4.

In the hybrid groups, the slower growing tumor seems to be more resistant to treatment and reached, respectively, 20.8, 41.7, 33.3, and 179.2

TABLE 4.—*Mammary carcinoma T. M. 8013, passage 191 in C57BL mice*

Group	Dose (γ)	Avg. weight of tumors* (*centigram*)	Controls (*percent*)	Avg. weight of spleen* (*centigram*)	Controls (*percent*)
Controls	—	86.7 ± 11.0	—	15.7 ± 0.8	
Actinomycin 616	5 × 2	7.7 ± 2.0	8.9	8.0 ± 2.9	50.9
C1		12.3 ± 2.4	14.2	9.0 ± 6.0	57.3
C2		11.3 ± 0.9	13.0	13.7 ± 2.9	87.3
C3		58.6 ± 14.8	67.6	13.0 ± 2.1	82.8
Actinomycin 616	5 × 1	115.0 ± 13.7	132.6	16.0 ± 0.6	101.9
C1		52.3 ± 23.6	60.3	14.0 ± 4.6	89.2
C2		76.0 ± 24.9	87.7	16.0 ± 1.5	101.9
C3		134.3 ± 45.1	154.9	16.7 ± 1.5	106.4

*± Standard Error of the mean.

percent of the controls for actinomycin 616, C1, C2, and C3. In 6 out of 8 experimental groups, tumors with a higher average weight than the control tumors were found when half the dose was given. These results suggest that a stimulation of tumor growth may occur when small doses of actinomycin are administered. The C3 fraction seems to be regularly less active than fractions C1 and C2 at the same dose level. The results of this experiment are shown in table 5.

TABLE 5.—*Mammary carcinoma T. M. 8013, passage 191 in (WLL × C57BL)F_1 mice*

Group	Dose (γ)	Avg. weight of tumors* (*centigram*)	Controls (*percent*)	Avg. weight of spleen* (*centigram*)	Controls (*percent*)
Controls	—	12.0 ± 0.6	—	11.0 ± 0.6	
Actinomycin 616	5 × 2	2.5 ± 0.5	20.8	4.5 ± 3.4	40.9
C1		5.0 ± 1.7	41.7	9.3 ± 0.9	84.5
C2		4.0 ± 1.3	33.3	10.0 ± 1.0	90.9
C3		21.5 ± 0.5	179.2	12.5 ± 2.6	113.6
Actinomycin 616	5 × 1	15.5 ± 3.4	129.2	12.5 ± 0.5	113.6
C1		19.5 ± 7.5	162.5	12.5 ± 0.5	113.6
C2		14.0 ± 1.2	116.7	12.7 ± 0.9	115.4
C3		59.3 ±16.2	494.2	16.7 ± 2.2	151.8

*± Standard Error of the mean.

Effect on Lymphosarcoma T. 24.179

This lymphosarcoma is a very malignant tumor obtained by Dr. Mühlbock of the Netherland's Cancer Institute. Implanted mice die after about 3 weeks with metastases in the spleen, liver, and lymph nodes, and usually with profuse ascites containing masses of neoplastic cells.

Forty female Odz mice, 5 months of age, obtained by mating O20 females with DBAb males without the mammary-tumor agent were implanted with a homogenate of 10-day-old tumors of passage 77, and separated into 5 experimental groups of 8 animals. The first group served as a control and the other groups were treated as in the first experiment with crude actinomycin 616 from Liège and its C1, C2, and C3 fractions. Sixteen days after implantation all mice were sacrificed. Before the end of the experiment all C1 treated mice died within 2 days, while in the other groups only 1 mouse succumbed.

The body-weight changes of the animals and the weights of tumors, metastatic lymph nodes, livers, and spleens are recorded in table 6.

The tumor, metastatic lymph nodes, and weight of the spleen and liver are remarkably influenced by treatment.

Histological examination revealed that the spleen and the liver of the control mice were full of metastatic cells; those from treated mice, who weighed much less, showed only beginning invasion of neoplastic cells.

Tumors from the actinomycin 616 treated group weighed 53 percent of controls, those from the C2 and C3 treated group, respectively, 23 and 45 percent. Metastases averaged 39, 17, and 31 percent of the controls.

TABLE 6.—*Lymphosarcoma T. 24.179, passage 78 on Odz (020 × DBAb)*

Group	Dose (γ)	Avg. weight change of animals (*gm.*)	Avg. weight of tumors* (*centigram*)	Controls (*percent*)	Avg. weight of metastatic lymph nodes* (*centigram*)	Controls (*percent*)	Avg. weight of spleen* (*centigram*)	Controls (*percent*)	Avg. weight of liver (*centigram*)	Controls (*percent*)
Controls.....	—	+5. 8	39. 0 ± 3. 4	—	24. 6 ± 4. 3	—	72. 1 ± 6. 2	—	190. 4	—
Actinomycin 616.....	5 × 2	+4. 9	20. 7 ± 4. 3	53	9. 7 ± 0. 8	39. 0	36. 7 ± 7. 2	51	120. 0	63. 0
C2.....	5 × 2	+2. 6	9. 0 ± 3. 0	23	4. 3 ± 0. 8	17. 0	12. 7 ± 0. 9	18	110. 2	57. 9
C3.....	5 × 2	2. 1	17. 6 ± 5. 4	45	7. 6 ± 0. 9	31. 0	25. 3 ± 3. 7	35	109. 3	57. 4

*± Standard Error of the mean.

Summary and Conclusions

By adsorption chromatography on alumina, and chromatography on cellulose, pure fractions (C1, C2, and C3; Co, Coa, and Cob) of a crude actinomycin were isolated. The fractions C1, C2, and C3 are biologically the most active. C2 is somewhat more toxic for mice and *Artemia salina* and exhibits slightly greater activity on *Bacillus subtilis* than fractions C1 and C3.

Actinomycin extracted from cultures of *Streptomyces* 616 has remarkable carcinostatic properties on a transplanted mammary carcinoma (T. M. 8013), and on a lymphosarcoma (T. 24.179) in mice. This activity seems to be in the fractions C1, C2, and C3. However, it is suggested that C3 is probably less active as a cytostatic agent than C1 and C2, administered at the same dose level. There is some evidence that low doses of actinomycin C may stimulate growth of carcinoma T. M. 8013.

The results of the tumor tests differ somewhat from those of the toxicity tests on mice and *Artemia salina* and also from the antibiotic activity on *Bacillus subtilis*. The toxicity of actinomycin C fractions parallel their antimicrobial properties but not completely their cytostatic activity.

Metastases in lymph nodes, spleen, and liver of the lymphosarcoma T. 24.179 are also influenced by actinomycin treatment but not completely cured or prevented, even when the first injection is given 24 hours after implantation. Further investigations may reveal whether preventive treatment with actinomycin C can have a curative effect.

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Fate of Skin Homografts in X-Irradiated Mice Treated With Homologous Marrow [1]

JOAN M. MAIN *and* RICHMOND T. PREHN,[2] *Laboratory of Biology, National Cancer Institute,*[3] *Bethesda, Maryland*

Lorenz, Congdon, and Uphoff (*1*, *2*) reported that mice could be protected from a lethal dose of X radiation by the injection of isologous [4] or homologous bone marrow immediately following X-radiation exposure. The reason for the sparing effect of the injected marrow was not immediately apparent and several hypotheses were advanced in explanation (*1–3*). One of these, the cellular repopulation hypothesis suggested that the injected or "grafted" marrow cells survived and subsequently repopulated the marrow of their recipients. This hypothesis was investigated by Lindsley (*4*), Merwin (*5*), and Ford *et al.* (*6*) who recently showed that homologous marrow cells may survive in X-irradiated hosts.

A previously reported study (*7*), motivated by the same hypothesis, showed that X-irradiated DBA/2 mice treated with bone marrow from (BALB/c × DBA/2)F_1 donors could be grafted successfully with skin from BALB/c mice. Since this result established a procedure for successful homografting between inbred strains it was considered advisable to investigate further the factors involved in the reaction. The present paper describes efforts along these lines. It was found that marrow dose, X-radiation dose, and the genetic relationship between marrow donor and recipient were the most critical variables in determining the fate of the homografts.

Experimental Procedures and Results

Techniques for X radiation, administration of bone marrow, and skin grafting have been described elsewhere (*1*, *7*, *8*). The dose of X radiation is given in the description of individual experiments. All bone marrow was injected intravenously and, unless stated otherwise, each mouse received marrow from humeri and femora of a single donor. All skin grafts were made 30 days after irradiation.

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[2] *Present address:* U. S Public Health Service Hospital, Seattle, Washington.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] Definition of terms: *isologous graft*, a transplant between animals of the same genotype; *homologous graft*, a transplant between animals of the same species but of different genotypes; *heterologous graft*, a transplant between animals of different species.

A skin graft was considered successful only after it had remained healthy for at least 3 months. A healthy graft retained at least half its original size, was covered with a luxuriant growth of hair, and was not inflamed.

All mice used in these experiments were from inbred strains obtained from the animal-production facilities at the National Institutes of Health, with the exception of strain C3H/He which was acquired from Dr. W. E. Heston. They were kept under similar environmental conditions in an air-conditioned room and fed Purina laboratory chow and tap water. The nomenclature (*9*) of the mouse strains is abbreviated in the text as follows: BALB/c for BALB/cAnN; DBA/2 for DBA/2JN; (L × A)F_1 hybrid for (C57L/HeN × A/HeNF)F_1 hybrid; (A × A)F_1 hybrid for (A/LN × A/HeN)F_1hybrid; C3H for C3H/He; and C57BL for C57BL/6JN.

Experiment 1

This experiment was designed to ascertain whether bone-marrow cells from an inbred strain will induce susceptibility to skin homografts in irradiated mice of a second inbred strain.

Forty-eight 3- to 10-month-old DBA/2 females each received 750 r of X radiation; 24 of these received bone marrow from BALB/c donors and 24 from DBA/2 donors. All mice received skin grafts from BALB/c donors. All marrow and skin-graft donors were 2- to 4-month-old males.

The results were as follows: Of 15 survivors that had received BALB/c marrow, 14 retained the skin grafts, whereas of 18 survivors that had received DBA/2 marrow, all shed the grafts within 20 to 80 days.

These results showed that BALB/c bone marrow induced susceptibility to BALB/c skin grafts in DBA/2 mice. Control DBA/2 mice that had been injected with isologous marrow were not susceptible to BALB/c skin grafts. This experiment extended earlier findings in which X-irradiated DBA/2 mice were successfully homografted with BALB/c skin after receiving marrow from (BALB/c × DBA/2)F_1 hybrids. However, the hybrid marrow possesses an advantage over the marrow from the inbred strain in that, theoretically, it is not stimulated immunologically by the antigens of the host, as well as by those of the homograft.

Trentin (*10*) has reported similar results with respect to survival of homologous skin grafts on X-irradiated mice protected by the same inbred strain marrow.

Experiment 2

The previous experiment showed that inbred mice receiving homologous marrow were successfully grafted with homologous skin from the marrow donor strain but that mice receiving isologous marrow did not retain the homologous skin grafts. These findings suggested that further efforts be made to test for the specificity of successful homografting between inbred mice.

This experiment was designed to test for specificity in mice that received homologous marrow by grafting them with skin from a third foreign inbred

animal, *i.e.*, with skin from mice homologous to both marrow donor and recipients. All homograft-hosts received 750 to 800 r of X irradiation. A variety of combinations was used and the results are summarized in table 1. DBA/2 mice receiving BALB/c marrow were not successfully grafted with C57BL skin grafts. However, 50 DBA/2 mice were not protected against a lethal dose of X radiation by C57BL marrow. All of these irradiated mice died from 7 to 45 days after receiving marrow.

In a second part of this experiment, irradiated F_1 hybrids were tested for susceptibility to homologous skins that were sewn together before grafting to the backs of the recipient hybrids. Irradiated (L $\times$ A)F_1 hybrids that had received BALB/c or DBA/2 marrow retained skin grafts from both strains, while none that received isologous hybrid marrow retained homografts from either strain. When irradiated (A $\times$ A)F_1 hybrids were injected with BALB/c marrow they retained a low percentage of homografts from BALB/c mice but only 1 homograft from strain C3H. However, when injected with strain C3H marrow they accepted only 1 homograft from a strain C3H animal.

The results permit the conclusion that when bone marrow from inbred mice is injected into mice of a second inbred strain, the recipients may or may not be susceptible to homografts from donors of a third inbred strain. Thus, in this experiment BALB/c marrow failed to render DBA/2 mice susceptible to C57BL skin grafts, but the same marrow enabled (L $\times$ A)F_1 hybrids to support DBA/2 skin grafts. It is probably significant that the BALB/c and DBA/2 strains are of the same histocompatibility-2 genotype, whereas, the BALB/c and C57BL are not.

Experiment 3

The persistence of the ability of successfully homografted mice to accept homologous skin grafts was investigated. Eighteen 3- to 10-month-old DBA/2 females were irradiated with 750 r, injected with BALB/c marrow, and grafted successfully with BALB/c skin from 2- to 4-month-old males. Three months later, the BALB/c homografts were removed and the area was replaced with skin from adult DBA/2 females. All retained their DBA/2 grafts. Two months later, the DBA/2 grafts were removed and the area was again replaced with skin from BALB/c males. The second BALB/c homografts were as successful as the first. Only 7 mice survived throughout the experiment, which lasted until 9 months after X irradiation.

These results showed that mice capable of accepting a homograft retained their ability to accept isologous skin and that after its growth they could again accept homologous skin.

Experiment 4

This experiment was designed to explore the relationship between the amount of homologous bone marrow injected and the survival of homografts.

TABLE 1.—*Experiment 2: Specificity of successful homografting between inbred mice*

Derivation of irradiated and protected mice*	No. of mice	Marrow donor†	Skin graft donor†	No. of surviving grafts / No. of surviving mice	Homograft survival (*percent*)
Strain DBA/2	50	C57BL	—	0	0
	26	BALB/c	C57BL	0/21	0
$(L \times A)F_1$ hybrid	28	BALB/c	BALB/c	19/24	79
			and DBA/2	9/24	37
	28	DBA/2	BALB/c	12/16	75
			and DBA/2	14/16	87
	34	$(L \times A)F_1$	BALB/c	0/28	0
			and DBA/2	0/28	0
$(A \times A)F_1$ hybrid	41	BALB/c	BALB/c	7/21	33
			and C3H	1/21	5
	44	C3H	BALB/c	0/13	0
			and C3H	1/13	8

*All marrow recipients were 3- to 10-month-old females.
†All marrow and skin donors were 3- to 10-month-old males.

A preliminary experiment had shown that simultaneous administration of DBA/2 and BALB/c bone marrow to X-irradiated strain DBA/2 mice made the irradiated mice susceptible to BALB/c skin grafts. In the present experiment, each DBA/2 mouse received marrow from a DBA/2 mouse on the assumption that the isologous marrow would afford radiation protection. For the purpose of the experiment the irradiated mice received different amounts of BALB/c marrow together with the DBA/2 marrow. All homograft hosts received X-radiation doses of 800 r and BALB/c skin grafts.

The experiment is summarized in table 2. Initially, almost all homografts healed well. However, in mice of the lower-dose groups, the homografts began to break down after several months. Usually the first sign of reaction was an inflammation of the entire graft, which was followed by foci of ulceration and gradual resorption of the entire graft. As seen in the table, approximately one third of the lower-dose mice lost their grafts within 5 months. Often, grafts that appeared inflamed recovered a healthy appearance at a later date. Some grafts underwent several cycles of "inflammation and recovery" but were still healthy after 250 days, at which time the experiment was terminated.

It is clear that the number of surviving homografts was in direct proportion to the amount of homologous bone marrow administered. Progressive decrease in the survival of grafts suggested that in these mice the homologous BALB/c marrow cells did not survive in their DBA/2 hosts.

Experiment 5

This experiment was performed to determine the relationship between dose of X radiation and homograft survival.

Three groups of 24 strain DBA/2 mice were X irradiated: one received 600 r; one, 300 r; and one, 100 r. All received BALB/c marrow and grafts of BALB/c skin. The mice were kept under observation for 120 days.

The results are shown in table 3. The percent survival of the homografts was in direct proportion to the amount of X radiation received. The dose of X radiation required for a high incidence of successful homografts was between 300 r and 600 r. It is emphasized that these results apply only to the conditions of this experiment, as different findings may accompany the use of different inbred strains and different doses of bone marrow. Many variables are probably involved in the reaction.

Experiment 6

In the preceding experiment, mice were exposed to 300 r of X radiation, injected with homologous bone marrow, and successfully grafted with homologous skin grafts. In the present experiment, irradiated mice received spleen instead of bone marrow to ascertain whether they also could be grafted with homologous skin.

Forty-eight 3-month-old strain DBA/2 females were exposed to 350 r of X radiation: 24 each received, intraperitoneally, a brei of spleen from 1

TABLE 2.—*Experiment 4: Relationship between dose of BALB/c bone marrow and homograft survival in X-irradiated DBA/2 mice**

Amount of BALB/c marrow injected†	No. of DBA/2 mice	No. of survivors grafted with BALB/c skin‡	Results after grafting at days: 30	60	90	120	150	250
Four bones (about 37.0×10^6 cells)	33	24	22/24§ *92*‖	22/24 *92*	21/23 *91*	21/23 *91*	20/22 *91*	19/21 *90*
One-half bone (about 17.0×10^6 cells)	47	45	40/43 *93*	40/43 *93*	34/40 *85*	29/38 *76*	26/37 *70*	25/36 *69*
One-quarter bone (about 2.0×10^6 cells)	27	27	25/27 *93*	24/27 *89*	22/26 *85*	17/26 *65*	17/26 *65*	12/26 *46*

*All graft recipients were 2- to 4-month-old females and each received 750 r of X radiation.
†Each mouse also received marrow from 1 DBA/2 donor.
‡All marrow and skin graft donors were 2- to 6-month-old males.
§ $\frac{\text{No. of surviving grafts}}{\text{No. of surviving mice}}$.
‖ Italicized figures = percent.

TABLE 3.—*Experiment 5: Relationship between dose of X radiation and BALB/c homograft survival* in strain DBA/2 mice protected with BALB/c marrow†*

X-radiation dose	No. of surviving grafts / No. of surviving mice	Homograft survival (*percent*)
600 r	22/23	96
300 r	7/24	29
100 r	0/24	0

*All marrow and skin-graft donors were male BALB/c mice 2 to 6 months of age.
†All homograft recipients were female DBA/2 mice 2 to 4 months of age.

(BALB/c × DBA/2)F_1 hybrid, and 24 controls received a similar spleen preparation from DBA/2 mice. All mice received skin grafts from BALB/c mice. The marrow and skin-graft donors were males, 2 to 6 months old.

The results were clear cut: 21 mice survived in each group; all homografts were successful in mice that received hybrid spleen and none were successful in those that received isologous spleen. Thus, under the conditions of this experiment, irradiated mice were homografted successfully after receiving homologous spleen cells.

Experiment 7

Results of the 2 preceding experiments showed that mice exposed to relatively low doses of X radiation were satisfactory test animals for homologous skin grafting. The purpose of this experiment was to determine the effectiveness of various routes of bone-marrow administration.

Strain DBA/2 mice were given 350 r of X radiation and then either BALB/c or (BALB/c × DBA/2)F_1 hybrid marrow was injected intravenously, intraperitoneally, or subcutaneously. Mice in each of the 3 groups were divided equally between those receiving marrow from the inbred strain and hybrids. Thirty days later all were grafted with BALB/c skin and kept under observation for 95 days.

The results are presented in table 4. The subcutaneous route of administration was the least effective in promoting successful homografts. These findings are not surprising because it is known that the route of antigen administration is important in inducing immunity to, or enhanc-

TABLE 4.—*Experiment 7: Response of DBA/2 mice* to BALB/c skin grafts following 350 r of X radiation and administration of BALB/c bone marrow by different routes†*

Route of marrow administration	No. of mice	No. of surviving grafts / No. of surviving mice	Homograft survival (*percent*)
Intravenous	56	33/38	87
Intraperitoneal	57	30/38	79
Subcutaneous	59	19/34	56

*All irradiated and protected mice were strain DBA/2 females, 2 to 9 months of age.
†All marrow and skin graft donors were BALB/c males, 2 to 6 months of age.

ing survival of, homologous skin homografts (*11*, *12*). No significant difference was found among mice that received marrow from the hybrid or from the inbred strain.

Experiment 8

This experiment was designed to determine whether X-irradiated mice grafted with homologous skin immediately after exposure to X-radiation could be successfully homografted 1 month later.

Forty-eight strain DBA/2 females, 2 to 8 months old, were exposed to 350 r of X radiation; 24 immediately received skin grafts and 24 immediately received bone marrow from BALB/c males. None of the skin grafts was successful. Thirty days later all survivors in both groups were given a BALB/c skin graft. All marrow and skin-graft donors were 2- to 6-month-old males.

The results were as follows: Of 19 mice that survived both BALB/c skin grafts, none accepted the second graft; whereas of 21 mice that survived after receiving BALB/c marrow and skin grafts, 16 accepted the skin grafts. These findings showed that the presence of a deteriorating homologous skin graft did not induce susceptibility to a second homologous skin graft. This experiment failed to confirm the observations of Hardin and Werder, who reported successful homografts in mice after exposure to 350 r of total-body X radiation (*13*).

Experiment 9

The first part of this experiment consisted of an effort to reveal whether homografted skin retained its ability to transplant when brought back to its strain of origin or had acquired an ability to grow in mice of the same strain as its foreign strain host.

The source of homografts was strain DBA/2 females that had carried successful grafts of BALB/c skin for at least 5 months. The homografts were removed and again grafted to untreated female DBA/2 or BALB/c recipients, 2 to 4 months of age. The experiment was terminated 90 days later.

The twice-transplanted BALB/c skin grafts were successful in 14 of 22 BALB/c recipients for an incidence of 64 percent. This incidence was lower than that usually obtained with isologous skin grafts in this laboratory, but the number of mice used was too small to permit any definite conclusion. However, there are several reasons why the lower incidence could be expected: (*a*) The antigenicity of the grafted tissues could have changed while residing in a foreign host; (*b*) the grafts could have acquired considerable DBA/2 stroma, which would resist proper vascularization in their new BALB/c hosts; or (*c*) the grafts could have lost their growth potential while residing in DBA/2 hosts.

The twice-transplanted BALB/c grafts gave interesting results in their foreign strain DBA/2 hosts. Eighteen of these DBA/2 mice were observed and, while none supported the homologous BALB/c tissue sufficiently to be recorded as a successful graft, in 9 mice about $\frac{1}{16}$ of the

grafted skin did survive. This reaction was probably the result of a small amount of DBA/2 tissue carried along in the graft, but the possibility remains that the BALB/c grafts had "adapted" to DBA/2 tissues (*14*) or that a subcellular enhancing factor was transferred in the skin (*15*).

The second part of this experiment was as follows: When the BALB/c homografts were removed from their DBA/2 hosts, another portion of DBA/2 skin was removed and grafted to other 2- to 4-month-old females of strains DBA/2 or BALB/c. This was done to ascertain whether contact with foreign tissues had altered the antigenic properties of the skin from DBA/2 hosts. The fact that the DBA/2 grafts "took" in 24 of 26 DBA/2 recipients and in none of 22 BALB/c recipients showed that, after exposure to X radiation, intravenous administration of BALB/c bone marrow, and contact with living BALB/c skin tissues for at least 5 months, the strain DBA/2 skin still retained its strain specificity.

Discussion

The present paper reports a series of exploratory experiments suggested by a previous observation that X-irradiated mice treated with homologous bone marrow were successfully homografted with skin from mice of a genetic constitution similar to that of the marrow donor.

In experiment 2, which concerned the specificity of this reaction, irradiated DBA/2 mice were injected with BALB/c marrow and tested for susceptibility to skin from C57BL donors. These C57BL grafts were unsuccessful. However, this test was not considered adequate for specificity because, in a simultaneous control, irradiated DBA/2 mice injected with C57BL marrow did not survive and thus, even with the present procedure for successful homografting, they could not be grafted with C57BL skin.

Additional tests for specificity with other genetic combinations showed that irradiated hybrids injected with homologous marrow frequently retained grafts of skin homologous to both marrow donor and recipient. However, it is emphasized that this experimental situation was designed to favor the survival of the homologous skin grafts. Since these irradiated mice were hybrids, they could have been grafted with skin from either parent strain (*17*). In addition, after exposure to radiation and injection with homologous marrow, they were susceptible to skin grafts from members of the marrow donor strain. Therefore these hybrids were theoretically susceptible to tissue grafts from 3 different inbred strains, each of which represented a variety of different antigens. The effects of any weak antigen present in a skin graft from a fourth inbred strain (but omitted from the other 3 strains) may have been overcome by residual X-irradiation effects.

In other experiments, the percentage of mice successfully homografted was found to vary directly with the X-radiation dose and dose of homologous marrow. Strain DBA/2 mice exposed to probable sublethal doses of X radiation were successfully grafted with BALB/c skin after injection with BALB/c marrow. In addition, irradiated DBA/2 mice

were successfully homografted after injection of combined marrow from BALB/c and DBA/2 donors. In these mice, a comparatively small amount of BALB/c marrow (from ¼ bone) was sufficient to induce susceptibility to homografts.

Probably a large percentage of the marrow cells in the successfully homografted mice in both of these experiments were of DBA/2 derivation. From a practical point of view, it would be interesting to determine the extreme lower limit of X radiation that would induce susceptibility to homografts employing massive doses of marrow. However, Trentin has recently found that the mortality in an experimental group of sublethally irradiated mice that received homologous marrow was increased over that in a control group of sublethally irradiated mice that did not receive marrow (*16*). Possibly the discrepancies between Trentin's results and ours can be accounted for by differences in genetic materials.

One of the most critical variables throughout these experiments was the genetic relationship among irradiated host, marrow doner and skin-graft donor. The present procedure for successful homografting may be limited to certain strain combinations because it is often dependent upon the ability of marrow from mice of the skin-graft donor strain to afford radiation protection to members of the graft-recipient strain. Caution should be used in extending the observations reported here to other genetic combinations, as strains BALB/c and DBA/2 both carry the *H-2*^{d} allele and may be of closely related genetic constitution. However, it has recently been shown that these two strains may differ by as many as 13 histocompatibility genes (*18*).

Summary and Conclusions

Experiments with mice homografted after X-radiation exposure and homologous bone-marrow injection have given the following results.

X-irradiated DBA/2 mice that had been treated with BALB/c marrow were successfully homografted with BALB/c skin. This experiment extended a previous observation, which showed that X-irradiated DBA/2 mice treated with (BALB/c × DBA/2)F_1 hybrid marrow were successfully homografted with BALB/c skin.

X-irradiated mice that had been treated with homologous marrow frequently retained skin grafts from mice homologous to both the marrow-donor and marrow-recipient strains.

Homologous skins were removed from successfully homografted mice. These mice accepted a second homograft of skin 2 months later.

The percentage of successful homografts between strains BALB/c and DAB/2 varied directly with the X radiation and marrow-cell doses.

Mice exposed to low doses of X radiation and injected with homologous spleen were homografted successfully 30 days later.

X-irradiated mice that had been injected intravenously or intraperitoneally with homologous marrow retained a higher percentage of homografts than mice injected with marrow subcutaneously.

Mice that had been X irradiated and grafted with skin immediately

after X-radiation exposure were not successfully homografted 30 days later.

Autogenous strain DBA/2 skin and strain BALB/c skin homografts, which were removed from successfully homografted DBA/2 mice and grafted to previously untreated DBA/2 or BALB/c recipients, were found unaltered in transplantability requirements.

Caution should be used in extending these observations to other genetic combinations as some of the strains used in these experiments carry the same allele at the histocompatibility-2 locus.

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Effects of Cell Density on Cell Growth in a Clone of Mouse Liver Cells [1,2]

DONALD M. PACE *and* LEFKOS AFTONOMOS, *Institute for Cellular Research, Department of Physiology, University of Nebraska, Lincoln, Nebraska*

In view of the paramount importance of the size of the original inoculum in studies on cells *in vitro*, it is essential that the range in inoculum size for optimum proliferation be determined for each new clone once it has been established.

Several studies have been conducted on the influence of the inoculum size on cell growth not only on tissue cells cultivated *in vitro* (*1–4*) but also on protozoan cells (*5–11*).

Earle *et al.* (*4*) used a clone of fibroblast cells (strain L) and determined the size of the cell inoculum necessary for maximum growth in a nutrient medium consisting of 40 percent horse serum, 40 percent balanced salt solution, and 20 percent (1:1) embryo extract. They reported that an initial 100,000 to 3,000,000 cells in 2 to 2.5 ml. of medium were adequate for proliferation; proliferation did not occur with 50,000 cells or less.

Survival of transplanted cells within a restricted inoculum-size range is associated with an adjustment of the nutrient fluid to the needs of the cells by the cells themselves. This adjustment or "conditioning" of the nutrient fluid to the needs of the cells may be accomplished either by a detoxification process along with other changes, such as redox potential adjustments (*12, 13*), or enzymes and autolytic products secreted by the cells (*14*), or secretion of one or more "growth-regulating substances" (*5–11*).

The cell *in vitro* is able to condition only a limited volume of its surrounding fluid. This is of great importance in establishing clones from a single cell, isolated from all other tissue cells. The cell will neither survive nor divide unless the volume of the surrounding fluid is reduced to a minute quantity. According to Sanford, Earle, and Likely (*13*), for strain L cells cultivated in horse serum-embryo extract medium this volume is approximately 400,000 μ^3. However, they find it impractical to maintain such a minute volume of fluid without loss by evaporation, change in the *p*H, and bacterial contamination. These obstacles are

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successfully overcome by the use of greater volumes of preconditioned medium.

Thus, for physiological studies upon cells *in vitro* it is necessary to know the critical limits of the inoculum-size range and also the effect of the conditioned nutrient fluid on the cells in question.

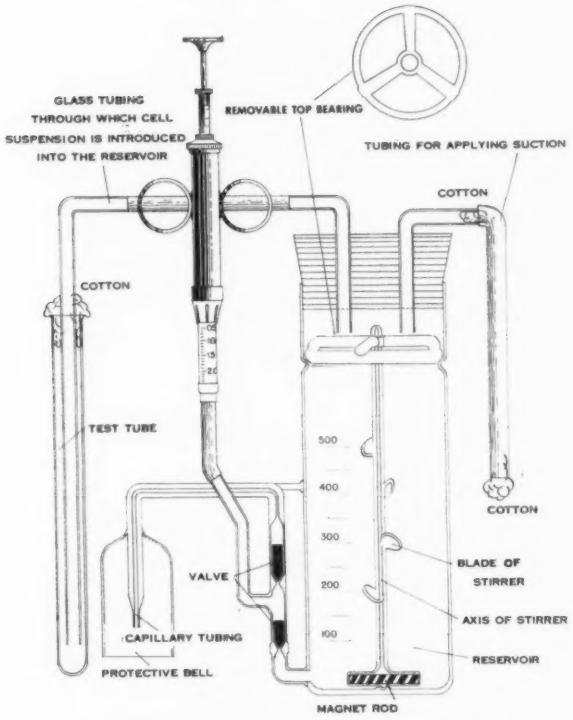
A clone of mouse liver cells, NCTC clone 1469, derived from a single cell in Earle's laboratory at the National Cancer Institute (*15*) has been cultivated in this laboratory for approximately 2 years. It is a cell type that proliferates rapidly and is useful for many physiological studies. However, nothing is known concerning the range of its optimum inoculum size; this should be established before further investigations are made.

The present work was carried out to determine: 1) The effect of the inoculum size on the rate of proliferation; 2) the minimum, optimum, and maximum inocula ranges; 3) the maximum number of cells obtainable in 1 ml. of nutrient fluid; and 4) the effect of different concentrations of conditioned medium on inocula below the minimum.

Materials and Methods

Replicate cultures of the liver cells in Carrel D-3.5 flasks were prepared from stock cultures cultivated in T-60 flasks. The stock cultures prior to use were examined macroscopically and microscopically. The nutrient fluid was removed from the flasks and the cells were washed gently with Earle's balanced salt solution; the washing was then repeated. Fresh nutrient fluid was introduced into the flasks and the cells were scraped from the bottom with a platinum microrake. The resulting aggregations of cells were further broken up by drawing them back and forth in a volumetric pipette. The suspension was then introduced into a pyrex-glass replicator (text-fig. 1) especially designed to keep the suspension uniform. By means of this replicator relatively large volumes of cell suspensions could be processed. Basically, the apparatus consists of a reservoir and a double-valve delivery spout to which is attached an improved B-D Cornwall spring return 2 ml. syringe equipped and calibrated to deliver 1 ml. of suspension into each Carrel flask. The cell suspension was kept uniform by means of a magnetic stirrer. This consisted of a glass rod with several glass blades attached about its length and a rod magnet surrounded by a pyrex-glass tube sealed in a horizontal position to the bottom of the stirring rod. This rod was held in a central position by means of a protrusion, or nipple, which fits into an indentation in the bottom of the reservoir. The nipple was large enough to hold the rod magnet off the floor of the reservoir in order to minimize crushing of cells by the grinding action of the rod.

The nutrient medium consisted of 40 percent horse serum, 40 percent Earle's balanced salt solution, and 20 percent (1:1) chick-embryo extract. A sufficient quantity of medium was made up at one time for use in all cultures of all horizontal series (tables 1 and 2). It was sterilized by filtration through a Selas filter of 03 porosity. During an experiment, the medium of each flask was replaced with fresh medium every 48 hours.



TEXT-FIGURE 1.—Replicator; apparatus used in the preparation of replicate cultures and in the renewal of the nutrient fluid of the cultures.[3]

Growth was measured by enumeration of cell nuclei by a modification of the method developed by Sanford *et al.* (*16*). For making nuclear counts, 3 cultures were selected at random for each experimental condition; they were pooled, mixed well, and 3 counts made for each. The counts were made every 48 hours immediately before renewing the fluid medium.

It is recognized that polynucleate cells may appear in cultures of this type, and therefore the nuclear count does not necessarily represent cell numbers. In this report, however, because of the insignificant number of polynucleate cells encountered, the nuclear count and cell count are used interchangeably.

In this investigation, replicate cultures with initial cell densities ranging from less than 90,000 to about 4,000,000 cells per ml. were prepared. At least 50 Carrel D-3.5 flasks were used for each density studied.

The cultures were incubated at 37.5° C. and observed until maximum cell numbers had been reached and a constant decline in growth established. This is readily ascertained by microscopic and macroscopic

[3] We are indebted to Mr. Walter Sampson, glassblower, of the Department of Chemistry, for his help in devising and making the replicator.

examination and nuclear counts. Photographic records of representative cultures were made at suitable time intervals.

Results

Cells in Fresh Medium

Several preliminary experiments revealed that proliferation did not occur to a significant extent if the original liver-cell densities were less than 90,000 or more than 4,000,000 cells per ml. of medium. Therefore, the major part of this study was made upon cultures inoculated with 17 different cell densities, ranging from 90,000 to about 4,000,000 cells per ml.

Nuclear counts were made every 2nd day. The results of these counts at the end of 8 days and at maximum cell population are presented in table 1.

TABLE 1.—*Relationship of original cell density to cell proliferation in fresh nutrient medium*

Inoculum size (*cells per ml.*)	Number of cultures	Percent of cultures showing proliferation*	Percent of cell population increase on the 8th day	Maximum cell population	Age at maximum cell population (*in days*)
90, 000	50	—	—	—	—
95, 000	100	50	1, 021	5, 210, 000	20
105, 000	50	100	844	5, 215, 000	16
155, 000	50	100	1, 320	5, 320, 000	14
189, 000	100	100	1, 683	6, 000, 000	16
263, 000	50	100	1, 432	5, 400, 000	14
348, 000	50	100	957	5, 500, 000	18
371, 000	50	100	1, 137	5, 500, 000	17
380, 000	50	100	1, 127	5, 400, 000	14
510, 000	50	100	621	5, 345, 000	16
630, 000	50	100	529	5, 560, 000	14
718, 000	50	100	567	5, 750, 000	16
727, 000	50	100	484	5, 750, 000	16
900, 000	50	100	412	5, 700, 000	15
1, 425, 000	50	100	286	5, 750, 000	9
3, 250, 000	50	18	65	5, 360, 000	8
4, 050, 000	50	6	—	4, 225, 000	10

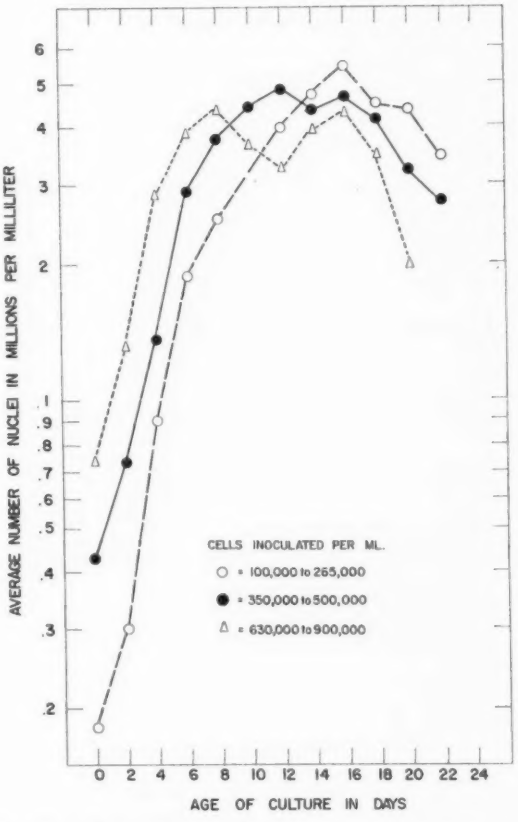
*Proliferation is said to occur if cell number becomes greater than the original.

It will be noted that good cell growth and reproduction are obtainable within a fairly wide range of original cell densities—from approximately 100,000 to 1,500,000 cells per ml. However, the rates of cell increase in cultures of cell densities greater than 500,000 progressively decrease from the lower to the higher inoculum values.

The maximum cell population per culture is not entirely independent of the original inoculum. As long as the cells survive and proliferate, the maximum cell population ranges from about 4.5 to 5.5 million cells per ml. in Carrel D-3.5 flasks. The lower maximum is obtained with the greater original cell densities.

Average rates of proliferation for a number of original cell densities within definite ranges were ascertained and plotted in terms of inoculum

sizes. Results for original cell densities ranging from approximately 105,000 to 263,000 cells per ml. are shown in graphic form in text-figure 2. The rate of proliferation increases progressively up until the 4th (perhaps between the 4th and 5th) day after inoculation. Increase in cell number continues until the 16th day and then rapidly declines; observations were not made after the 22nd day.



TEXT-FIGURE 2.—The effect of initial cell number on growth in liver cells of NCTC clone 1469.

Average rates of proliferation in cultures with original cell densities between 348,000 and 510,000 are also presented in text-figure 2. The rate of proliferation increases progressively until about the 6th day. There is a continued increase in cell number until the 12th day, after which there is a decrease.

The third curve in text-figure 2 represents the average nuclear counts in cultures that had been inoculated with cells ranging in number from 630,000 to 900,000 per ml. With such high initial densities, two peaks

appear on the curve. The first peak represents the rate of growth for the first 8 days after which many of the cells, because of crowding, became necrotic. The second peak—about as high as the first one—appears on the 16th day after inoculation. This also represents a period of rapid growth and evidently occurs because the medium was changed after the number of cells had decreased considerably. Thus, the dead and weakened cells probably were washed out of the cultures leaving behind the stronger, healthier cells, which began proliferating again.

Cells in Conditioned Medium

All the cultures used in the work reported above contained fresh medium and any culture inoculated with less than 90,000 cells per ml. did not proliferate nor survive for any great length of time. The question arises as to whether or not the same would be true if conditioned media were used in place of fresh. With this in mind, several experiments were carried out to compare rates of growth in cells cultivated in a fresh medium with others in a medium in which cells had already grown; this latter medium was considered to be conditioned. The conditioned medium was obtained from actively proliferating 48-hour cultures; glucose (1 gm. per l.) was added to this medium; then, the medium was filtered to ensure that it was cell-free.

The results are presented in table 2 and agree with previous findings indicating again that in fresh nutrient medium, if the original cell density of a culture is 90,000 cells per ml. or less, there is no proliferation.

In the conditioned medium, there was an increase in cell numbers even in cultures in which only 5,000 cells per ml. were inoculated. However, this was true only for the first 7 days; they died out between the 7th and 14th days. The cells grew well in cultures with original densities of 10,000, 20,000, and 40,000 cells but after 14 days, when fresh medium was added, they also died.

Very good growth was observed in the conditioned medium to which 70,000 or 90,000 cells per ml. were added and after the 14th day, when the conditioned medium was replaced by fresh solution, they continued to proliferate in a normal manner.

Growth in cultures consisting of 50 percent fresh and 50 percent conditioned medium were also observed and, although some proliferation occurred during the first 7 days in some of these cultures, the cells began to disappear during the 2nd week of the experiment.

Discussion

Many attempts have been made to cultivate single tissue cells *in vitro* but they were unsuccessful until Sanford, Earle, and Likely (*13*) isolated single cells in very limited volumes of nutrient medium. Several suggestions have been made to explain this phenomenon. The most logical explanation postulates the production of a growth-promoting substance by the cells themselves. The effect of such a "growth substance" would

TABLE 2.—*Comparison of growth of liver cells in fresh nutrient medium and in conditioned medium when different numbers of cells were added**

Designation of medium	Number of cells inoculated per ml.	Number of cultures	Number of cells (*7th day*)	Number of cells (*14th day*)	Percent increase (*by 14th day*)	Percent of cultures proliferating (*up to and beyond 14th day*)
Fresh medium, 1W	5,000	30	none	—	—	—
Fresh medium, 3W	5,000	30	none	—	—	—
Conditioned medium, 1W	5,000	30	7,000	none	—	—
Conditioned medium, 3W	5,000	30	5,000	none	—	—
Fresh medium, 1W	10,000	30	none	—	—	—
Fresh medium, 3W	10,000	30	none	—	—	—
Conditioned medium, 1W	10,000	30	12,000	25,000	150	100
Conditioned medium, 3W	10,000	30	8,000	15,000	50	100
Fresh medium, 1W	20,000	30	none	—	—	—
Fresh medium, 3W	20,000	30	none	—	—	—
Conditioned medium, 1W	20,000	30	25,000	50,000	150	100
Conditioned medium, 3W	20,000	30	18,000	40,000	100	100
Fresh medium, 1W	40,000	30	none	—	—	—
Fresh medium, 3W	40,000	30	none	—	—	—
Conditioned medium, 1W	40,000	30	50,000	90,000	125	100
Conditioned medium, 3W	40,000	30	35,000	60,000	50	100
Fresh medium, 1W	70,000	30	none	—	—	—
Fresh medium, 3W	70,000	30	none	—	—	—
Conditioned medium, 1W	70,000	30	85,000	280,000	300	100
Conditioned medium, 3W	70,000	30	72,000	150,000	110	100
Fresh medium, 1W	90,000	30	21,000	15,000	none	—
Fresh medium, 3W	90,000	30	11,000	5,000	none	—
Conditioned medium, 1W	90,000	30	150,000	500,000	450	100
Conditioned medium, 3W	90,000	30	120,000	380,000	320	100

*Glucose (1 gm. per l.) was added to the conditioned media; 1W, medium renewed weekly; 3W, medium renewed every 48 hours.

undoubtedly be determined by the concentration of the substance within the cytoplasm, and this concentration, of course, depends on the concentration gradient existing at the time between the cytoplasm and its external environment.

It has been established that the liver cells used in this study grow well if original cell densities are 100,000 per ml. or greater. This suggests that 100,000 cells, bathed in 1 ml. of nutrient medium, condition the medium so that the cells survive; in other words, 1 (average) cell will condition 0.01 mm.^{3} of medium. The average liver cell of this clone has a diameter of 21 μ; each cell therefore has a volume of approximately 4,850 μ^3. (The cells were placed on a micrometer slide, photographed so that a reproduction of the scale appeared along with the cells, and then measured.) Thus, a single average cell is able to condition a volume of medium approximately 2,000 times its own volume.

It may be assumed that a growth substance normally may diffuse from within the cell into the surrounding fluid at a rate that could maintain a proper balance between the concentrations inside and outside, and thus avoid subminimal concentrations within the cell. Evidently, if the ratio of fluid volume to liver-cell volume is greater than 2,000 : 1, the diffusion rate of substances to the outside of the cell may be so great that subminimal concentrations would soon be reached within the cell; the cell then fails to reproduce and eventually dies. These are essentially the facts as obtained in earlier investigations on the production of growth-regulating substances by protozoan cells (*5–11*).

In the greater original cell densities (1,425,000 cells per ml. and above) that will support cell proliferation satisfactorily, the volume of the surrounding medium per cell is reduced to approximately 0.0007 mm.^{3}; this is about 145 times the volume of the average cell. If the volume of the medium is reduced further, proliferation may not occur. This indicates that the rate of diffusion at these volume ratios is decreased to such an extent that the concentration of the growth substance within the cell increases. These high concentrations may have an inhibiting effect that could prevent cell growth and cell increase. At any rate, the volume of the fluid is a limiting factor. It is logical to expect a smaller volume to become saturated more rapidly with growth-regulating substances or, if these did not exist, with wastes and other products of cell metabolism; also its energy sources would be exhausted sooner than in a larger volume containing the same number of cells.

If we accept the statements above as true along with the fact that the maximum number of liver cells in 1 ml. of nutrient is between 5,000,000 and 6,000,000 (in D-3.5 Carrel flasks), it seems logical to assume that the concentration of the growth substance within the cell will be critical and lethal if the ratio of nutrient fluid volume to average cell volume is 30:1 or less.

Although inocula ranging from 95,000 to 4,000,000 cells per ml. may survive and proliferate, the optimum inoculum range is between approximately 150,000 and 400,000 cells per ml., for the following reasons:

1) Cultures inoculated with cells so as to give a density within the optimum range show a 100 percent survival; the daily rate of proliferation is high during the first week and shows a gradual decrease after this period.
2) The daily rate of proliferation of cells within this optimum range of inoculation is the highest of all during the first week. This indicates that these cultures are well established soon after inoculation and that the concentration of any growth-regulating substance within the cells is optimum or at least is present at such concentrations as to compensate for slight injuries suffered by the cells on removal from the mother culture.
3) Cultures inoculated with a greater number of cells than the optimum range of inoculation show a slower daily rate of proliferation than cultures inoculated with cells within the optimum range.
4) Cultures inoculated with cells lower in number than the optimum range (but not lower than the minimum) show an increasingly higher daily rate of proliferation until maximum cell population is reached. This indicates that the cells in the cultures are still establishing themselves during the 2nd week after transplantation.

These tests show that the maximum daily rate of proliferation in intact cultures occurs when the population has reached approximately 900,000 cells per ml. or, in this case, per culture. This margin is reached in cultures with initial densities less than 190,000 cells per ml. on or after the 8th day following inoculation. For cultures within or above the optimum range of inoculation this margin is reached around the 3rd day after inoculation; by the 8th day the cell population in these cultures has reached 60 to 80 percent of the maximum cell population. At this point crowding causes a decline in the daily rate of proliferation.

Another indication that these cells may produce growth substances is found in the behavior of cells in the cultures, started with subminimum number of cells in a conditioned medium. The results can be explained on the basis of differences in diffusion rates of growth substances between the cells and the surrounding medium. Conditioned medium, which presumably already contains a certain concentration of growth substance, will regulate the rate of diffusion so that the concentration of the growth substance within the cell will remain within the optimum limits. This, perhaps, is the reason that cells survive and proliferate in cultures with initial cell densities as low as 10,000 cells per ml.

Summary

1) Using a clone of mouse liver cells (NCTC clone 1469), a study has been made on the effects of the initial cell density on growth and reproduction of these cells in Carrel D-3.5 flasks.

2) Initial cell density ranged from 5,000 to 4,000,000 cells per ml.

3) In fresh medium the optimum range of inoculum size is approximately between 150,000 and 380,000 cells per ml. Inocula below 90,000 and above 4,025,000 cells per ml. did not survive transplantation; cultures with inocula between 90,000 to 105,000 and between 1,425,000 to 4,025,000 per ml. are inconsistent as to survival and proliferation.

4) The rate of proliferation is affected by the size of the original inoculum only during the 1st week of cultivation *in vitro*. During this time the rate of proliferation of cultures inoculated with optimum densities is the highest of all.

5) The maximum cell population per culture depends chiefly on the area of the substrate and the volume of the fluid used, and not on the initial cell density. In these experiments, using D-3.5 Carrel flasks and 1 ml. nutrient medium, the maximum population is between 5,400,000 and 6,000,000 per flask.

6) By using a conditioned medium, with 1 gm. per l. of glucose added, proliferation occurs with an original inoculum as low as 10,000 cells per ml. In this conditioned medium the frequency of the renewal of the medium influences the cells; the cells in the fluid that is renewed only once a week do better than those in which the fluid is renewed every 48 hours.

7) Cells which are cultivated in the conditioned medium and which reach a population of 90,000 cells per ml. by the end of the 2nd week, survive and proliferate normally if their medium is replaced with fresh medium at this time and changed every 48 hours.

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Inhibitory Effect of Some Aminoazo Dyes and Other Carcinogens on Glutamate Oxidation in Mitochondria of Riboflavine-Deficient Rat Liver [1]

RUTH K. KIELLEY, *Laboratory of Biology, National Cancer Institute,*[2] *Bethesda, Maryland*

The outstanding metabolic characteristic of all cancer cells is their tendency to display a high glycolytic activity. At the same time, many types of cancer cells display a lower respiratory activity than many normal ones. Observations of this kind from his own early work (*1*) and from numerous subsequent investigations (*2–5*) form the basis for Warburg's belief that the primary cause of cancer is an irreversible injury of respiration. In a recent article (*6*), Warburg has reaffirmed and elaborated his original views in relation to more recent developments on ascites-tumor cells, cell particulates, and tissue culture. His concept is, briefly, that in the development of cancer, the energy deficiency resulting from injury of respiration is gradually compensated for by increased glycolysis. Only those cells that can, by mutation or modulation, fully compensate for the energy deficiency in this manner become cancer cells. Weinhouse (*7*) has reviewed and critically examined the whole experimental basis for Warburg's belief that impairment of respiration is the primary cause of cancer. Although one may agree with Weinhouse that there is considerable evidence which does not support such a view, nevertheless, the basic concept has not been disproved and continues to challenge the imagination and resources of those engaged in the problem of carcinogenesis.

In a consideration of Warburg's views, it seemed that if irreversible impairment of respiration is indeed the *primary cause* of cancer, then in some instances at least, direct inhibition of respiration by carcinogens may be a *primary reaction* in the first phase of carcinogenesis. In this event, one might expect that certain carcinogens would have some more or less direct inhibitory effects on specific enzymes of mitochondria, the principal site of respiratory activity in the cell (*8*). Such a direct experimental approach has undoubtedly occurred to many biochemical investigators, but very few studies on the *in vitro* effect of carcinogens on mitochondrial oxidations have been reported (*9, 10*).

Recently, in this laboratory, the possibility of observing some *in vitro* inhibitory effects of chemical carcinogens on the respiration of intact

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

liver mitochondria was explored. In the first experiments which were reported (*11*), it was found that glutamate oxidation by phosphorylating mitochondria of rat liver was markedly inhibited by the carcinogen 2FdiAA,[3] whereas other pyridine nucleotide-linked oxidations (α-ketoglutarate, *d*-isocitrate, and *dl*-β-hydroxybutyrate) were considerably less inhibited, and succinate oxidation was scarcely affected. After other fluorene derivatives were tested as inhibitors, a rough correlation was seen between the potency of various fluorene compounds as carcinogens and the degree to which glutamate oxidation was inhibited. In studies on the mechanism of inhibition (*12*, *13*), it was shown that the glutamic dehydrogenase of mitochondria was inhibited by the fluorene carcinogen which appeared to act as a powerful competitor of DPN for the enzyme. The inhibition phenomenon was abolished by treating the mitochondria with the uncoupling reagent, 2,4-dinitrophenol. In a recent note, Emmelot (*14*, *15*) has confirmed the observation on inhibition of glutamate oxidation in mouse liver mitochondria with the carcinogen 2FdiAA and has also observed that the inhibition can be prevented by addition of DPN.

Other classes of carcinogens, especially the aminoazo dyes, have since been investigated in this laboratory for possible inhibitory effects on glutamate oxidation by mitochondria of rat and mouse liver. In this paper the results of some studies on the response of normal and riboflavine-deficient mitochondria of rat liver to some aminoazo dyes and other carcinogens are presented. The results show that inhibition of glutamate oxidation was greatly increased in riboflavine-deficient mitochondria and that inhibition by the carcinogen DPAA, which was not seen with normal mitochondria, was significant under these conditions.

Materials and Methods

Male rats of the Sprague-Dawley strain weighing about 200 to 250 gm. were used. Groups of 4 animals were fed the following diets for a period of 10 to 14 weeks: (a) the stock diet of Purina chow pellets, (b) a semisynthetic basal diet deficient in riboflavine,[4] and (c) the same basal diet supplemented with riboflavine.[4]

Homogenates of liver were prepared in 0.25 *M* sucrose, and mitochondria were isolated by methods previously described (*16*, *17*). Oxygen

[3] The following abbreviations are used in this paper: 2FdiAA for *N*-2-fluorenyldiacetamide; DPN for diphosphopyridine nucleotide; DPAA for *N,N*-dimethyl-*p*-phenylazoaniline, formerly 4-dimethylaminoazobenzene (DAB); RF for riboflavine; DTAA for *N,N*-dimethyl-*p*-(*m*-tolylazo)aniline, formerly 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB); DPAT for *N,N*-dimethyl-*p*-phenylazo-*m*-toluidine, formerly 2-methyl-4-dimethylaminoazobenzene (2MeDAB); TAT for 4-*o*-tolylazo-*o*-toluidine, formerly *o*-aminoazotoluene (AAT); ATAT for *N*-acetyl-*p*(*o*-tolylazo)*o*-toluidine, formerly *N*-acetyl derivative of *o*-aminoazotoluene (AAAT); PAA for *p*-phenylazoaniline, formerly *p*-aminoazobenzene (AB); CCl_4 for carbon tetrachloride; MC for 3-methylcholanthrene, formerly 20-methylcholanthrene.

[4] The basal diet deficient in riboflavine was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and had the following composition: 18% "vitamin free" casein, 68% sucrose, 10% vegetable oil, 4% U.S.P. salt mixture #2, and a vitamin fortification mixture lacking riboflavine. The formulation of the vitamin fortification mixture was as follows: (gm./100 lbs. diet) vitamin A concentrate (200,000 units per gm.) 4.5, vitamin D concentrate (400,000 units per gm.) 0.25, α-tocopherol 5.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, *p*-aminobenzoic acid 5.0, niacin 4.5, pyridoxine hydrochloride 1.0, thiamin hydrochloride 1.0, calcium pantothenate 3.0; (mg./100 lbs. diet) biotin 20, folic acid 90, vitamin B_{12} 1.35. The basal diet supplemented with riboflavine contained 1 gm. of riboflavine per 100 lbs. of diet.

uptake was determined manometrically. Phosphate uptake was determined by the disappearance of inorganic phosphate as measured by the Fiske-Subbarow method. DTAA and DPAT were kindly supplied by Dr. J. A. Miller of the McArdle Memorial Laboratory for Cancer Research. Other aminoazo compounds and MC were highly purified preparations. Additional materials used in this study were those previously described (*12*, *13*).

Results

Mitochondria from livers of rats maintained on the stock diet of Purina chow pellets were resistant to the inhibitory action of most of the carcinogens and all of the noncarcinogens tested in the glutamate system. As seen in a typical experiment (table 1), the fluorene carcinogen 2FdiAA almost always produced moderate inhibition. The aminoazo carcinogen TAT, likewise, produced in this case moderate inhibition, but its effect was variable, often exerting little or no inhibition. The potent liver carcinogen DTAA showed a slight but consistent inhibitory effect on glutamate oxidation, but in no case was there any significant inhibition by the carcinogen DPAA.

TABLE 1.—*Inhibition of glutamate oxidation by various carcinogens and related compounds in riboflavine-deficient and normal mitochondria of rat liver**†*

Compound	Purina chow diet		RF-deficient diet		RF-supplemented diet	
	O_2 uptake (*μl.*)	Inhibition (*percent*)	O_2 uptake (*μl.*)	Inhibition (*percent*)	O_2 uptake (*μl.*)	Inhibition (*percent*)
None	51	0	53	0	50	0
CCl_4	50	2	24	55	47	6
2FdiAA	38	26	22	58	37	26
DPAA	49	4	32	40	51	0
DTAA	45	12	19	64	41	18
PAA	48	6	32	40	45	10
TAT	39	24	24	55	29	42
ATAT	50	2	49	8	48	4
DPAT	51	0	23	57	29	42
MC	54	0	53	0	51	0

*The results are expressed as μl. of oxygen taken up by mitochondria equivalent to 100 mg. of liver.

†The reaction vessels contained 0.05 *M* histidine, 0.005 *M* $MgCl_2$, 0.0167 *M* KH_2PO_4-K_2HPO_4, 0.0069 *M* adenosine-5′-PO_4, 1.33×10^{-3} *M* cytochrome *c*, 0.038 *M* KCl, 0.035 *M* K glutamate, 0.025 ml. of ethanol or 0.025 ml. of 6×10^{-3} *M* carcinogen dissolved in ethanol‡, 0.2 ml. of mitochondria and water to make a final volume of 1.5 ml.–*p*H 7.0, temperature 28° C., equilibration period, 10 minutes, and time, 15 minutes.

‡DPAA and to a lesser extent DTAA and DPAT tended to separate out. The final concentration of these substances in the incubation medium therefore was considerably less than 1×10^{-4} *M*. In the case of CCl_4, the stock solution prepared just before use contained 0.5 ml. of CCl_4 and 9.5 ml. of ethanol.

Under conditions of riboflavine deficiency, however, the inhibition picture was radically different as shown in table 1. All of the aminoazo carcinogens as well as 2 noncarcinogens of this class, PAA and DPAT, inhibited glutamate oxidation very markedly. In addition, inhibition by CCl_4, which was not observed with normal mitochondria, was now extensive and that by 2FdiAA was greatly increased. The only compounds that had no significant inhibitory effect were ATAT which is

stated to be less carcinogenic than TAT[5] and MC which is not known to produce liver tumors. Inhibition by DPAA appeared to depend upon the extent of riboflavine deficiency developed in the rat as judged by external symptoms of nervousness and porphyrin secretion; the more severe the deficiency, the greater was the inhibition (*cf.* inhibition by DPAA in table 1 and table 2). A point of some interest is that in riboflavine-deficient mitochondria, there appeared to be no great lack of DPN for glutamate oxidation.

Some differences in the inhibitory response to various compounds were found between mitochondria from rats fed the chow diet and those from rats fed the semisynthetic basal diet supplemented with riboflavine. In table 1 it is seen that mitochondria of both groups responded similarly except to TAT and DPAT. The former mitochondria were either not inhibited or only moderately inhibited by TAT and generally not inhibited by DPAT while the latter mitochondria were either moderately or rather strongly inhibited by both aminoazo dyes.

The inhibition of glutamate oxidation by various carcinogens and related compounds raised the question of whether the oxidations remain coupled to phosphorylation in the presence of inhibitor. The results in table 2 show that in every case except CCl_4, the values were in the range 2.4 to 3.2, indicating that these inhibitors did not cause any extensive uncoupling. These results also suggest that the inhibition is primarily one on oxidation. CCl_4, in the concentration used, appeared to be an exception in causing considerable uncoupling of phosphorylation from oxidation.

TABLE 2.—*Effect of various carcinogens and related compounds on oxidative phosphorylation in riboflavine-deficient and normal mitochondria of rat liver**†*

Compound	RF-deficient mitochondria			Normal mitochondria		
	O_2 uptake (*µatoms*)	P uptake (*µM*)	P/O	O_2 uptake (*µatoms*)	P uptake (*µM*)	P/O
None (control)	7.0	17.7	2.5	7.1	17.6	2.5
CCl_4	4.2	6.5	1.6	6.8	10.6	1.6
2FdiAA	3.4	10.8	3.2	5.3	15.4	2.9
DPAA	6.0	16.9	2.8	7.4	17.7	2.4
DTAA	4.2	13.5	3.2	5.8	15.5	2.7
PAA	3.9	12.5	3.2	6.4	16.2	2.5
TAT	4.2	11.7	2.8	4.2	13.5	3.2
ATAT	7.2	17.7	2.5	6.9	17.7	2.6
DPAT	3.6	11.5	3.2	4.1	12.8	3.1
MC	7.5	17.8	2.4	7.4	18.0	2.4

* The conditions were those given in table 1 except that the total incubation time was 30 minutes, including 5 minutes for equilibration. Oxygen uptake during the equilibration period was assumed to be equal to that measured during the first 5 minutes. To stop the reaction, 0.3 ml. of 15% perchloric acid was added from the side arm of the flask.

†Oxygen and P uptake by mitochondria equivalent to 100 mg. of liver.

[5] According to Kinosita (*18*), the mono- and di-acetylated derivatives of TAT can produce hepatic changes similar to those produced by the parent compound, but the more they are acetylated, the weaker is the carcinogenic effect. - It might be inferred from this that ATAT is not carninogenic and that deacetylation to TAT is necessary for tumor induction.

Inasmuch as some noncarcinogens as well as carcinogens inhibited glutamate oxidation quite markedly under certain conditions, it was of interest to study their effect on the oxidation of other substrates in order to see whether the inhibition pattern of carcinogens is different from that of noncarcinogens. Three aminoazo compounds, including one carcinogen (DTAA) and two noncarcinogens (PAA and DPAT), were tested against various oxidizable substrates for inhibitory effects on riboflavine-deficient mitochondria. Similarly, TAT (a carcinogen) and DPAT (a noncarcinogen) were tested on "normal" mitochondria from rats fed the basal diet supplemented with riboflavine. The results are shown in table 3. In the tests with riboflavine-deficient mitochondria, it is seen that all three aminoazo compounds inhibited glutamate oxidation strongly, the largest effect being seen with the noncarcinogen DPAT. The oxida-

TABLE 3.—*Effect of some aminoazo compounds on the oxidation of various substrates by mitochondria of riboflavine-deficient and normal rat liver**†*

Oxidizable substrate	RF-deficient mitochondria				Normal mitochondria		
	Control (μl.)	DTAA (μl.)	PAA (μl.)	DPAT (μl.)	Control (μl.)	TAT (μl.)	DPAT (μl.)
L-Glutamate	85	36	30	18	84	67	53
d-Isocitrate	59	45	37	32	51	26	33
α-Ketoglutarate	23	20	25	20	21	18	18
dl-β-OH-Butyrate	14	15	15	16	25	24	26
Succinate	153	165	155	117	177	152	100

*Substrate concentrations were as follows: 0.035 *M* glutamate, 0.02 *M* *d*-isocitrate, 0.02 *M* α-ketoglutarate, 0.02 *M* *dl*-β-hydroxybutyrate, 0.05 *M* succinate. Equilibration time 12 minutes; time 20 minutes, 0.05 ml. to 0.3 ml. of mitochondria.

† Results are expressed as μl. of oxygen taken up by mitochondria equivalent to 100 mg. of liver.

tion of isocitrate was moderately inhibited by the carcinogen and somewhat more so by the noncarcinogens. The oxidation of α-ketoglutarate was slightly inhibited by DTAA and by DPAT while β-hydroxybutyrate oxidation was not inhibited by any of the compounds. It is noteworthy that succinate oxidation was inhibited only by DPAT. In the case of normal mitochondria, the results were similar to those obtained with riboflavine-deficient mitochondria except that inhibition of glutamate oxidation was not so great.

Discussion

The results of the present studies on the effect of some aminoazo dyes on glutamate oxidation by mitochondria indicate that, under certain nutritional conditions, mitochondria isolated from rat liver may be quite susceptible to the inhibitory action of some carcinogenic and noncarcinogenic compounds. The appearance of considerable inhibition of glutamate oxidation by DPAA (40%) in riboflavine-deficient mitochondria stands in sharp contrast to the fact that significant inhibition by DPAA was never observed with normal mitochondria. These results are in agreement with the well-known observation that a high incidence of liver tumors

in rats is dependent on a low riboflavine level in the diet (*19*, *20*),[6] but it does not preclude the possibility that other nutritional deficiencies may also favor inhibition of glutamate oxidation by DPAA. Emmelot (*14*, *15*) has recently reported that mitochondrial (mouse liver) oxidation of glutamate was not inhibited by DPAA, although it was inhibited by *N*-methyl-*p*-phenolazoaniline (4-monomethylaminoazobenzene), TAT, and PAA. In the present work, DTAA, which was found by Miller and Baumann (*21*) to be a more potent carcinogen in the rat than DPAA, inhibited glutamate oxidation in riboflavine-deficient mitochondria more strongly than did DPAA and often exerted some inhibitory effect on normal mitochondria. On the other hand, TAT which is considered to be a weak carcinogen in the rat (*20*) was a more potent inhibitor of glutamate oxidation than DTAA. Furthermore, PAA which is regarded as noncarcinogenic and DPAT also a noncarcinogen (*20*) were found to be effective inhibitors under certain conditions.

The inhibitory effect of carcinogenic and noncarcinogenic aminoazo compounds, alike, on glutamate oxidation might lead one to conclude that if these inhibitory effects have any counterpart in the living cell, they are manifestations of toxicity and that the difference between toxicity and carcinogenic effects cannot be distinguished. However, there is some evidence that the inhibition pattern of mitochondrial oxidations shown by some toxic noncarcinogenic substances may be different from that shown by carcinogens. All of the carcinogenic compounds that produce liver tumors which have been investigated so far, including the fluorene carcinogens (*11*), have an inhibition pattern that is remarkably similar. In this pattern glutamate oxidation is most markedly inhibited, *d*-isocitrate oxidation is moderately to strongly inhibited, α-ketoglutarate and β-hydroxybutyrate oxidations are slightly or not significantly inhibited, and succinate oxidation is not significantly inhibited. The inhibition pattern of the noncarcinogen DPAT differs from that of carcinogens in that there is a significant inhibition of succinate oxidation. Because this inhibition was often only moderate (24%) or moderately large (40%), experiments with DPAT were repeated several times with different mitochondrial preparations. The results are consistent in showing that DPAT causes a significant inhibition of succinate oxidation in both normal and riboflavine-deficient mitochondria. This property of DPAT may possibly be related to the observation that although the compound is quite toxic to rats, no tumors develop and there is seen in the liver tissue, perhaps as the result of some compensation mechanism for overcoming the inhibition, a large increase in the number of mitochondria with a corresponding increase in succinoxidase activity (*22*, *23*). In the case of PAA, the large inhibitory effect seen in riboflavine-deficient mitochondria and the finding that the inhibition pattern of this compound corresponds to that of carcinogens are apparent inconsistencies with the noncarcinogenic nature of this dye. However, the anomaly may suggest that PAA, although not generally regarded as a carcinogen (*20*), is in-

[6] For an extensive review on carcinogenesis by aminoazo dyes, *see* Miller and Miller (*20*).

trinsically toxic and that given a certain combination of conditions including a high concentration of dye approaching toxic levels, it may be carcinogenic (*24*). As already indicated in table 1, the true concentration of the more insoluble aminoazo dyes such as DPAA may be something far less than is calculated from the amount of DPAA added to the reaction mixture. PAA, on the contrary, is quite soluble under these conditions so that its actual concentration might have been several times greater than the concentration of many of the carcinogens tested. It is possible that under the usual conditions of producing liver tumors in rats, the rate of inactivation of PAA [7] is sufficiently great to prevent the appearance of tumors. Likewise, differences in the rates of modifying reactions in the rat and mouse could account for the differences in carcinogenicity of TAT in these animal species.

Of the nonaminoazo compounds tested in this study, CCl_4 caused extensive inhibition of glutamate oxidation whereas MC did not. CCl_4 causes liver tumors in mice (*31*), but attempts to produce tumors in the rat with this substance have not been successful (*20*). It is of interest in this connection that CCl_4 inhibited glutamate oxidation only in rat liver mitochondria that were deficient in riboflavine whereas in mouse liver mitochondria extensive inhibition by CCl_4 was observed in every preparation. The results on mouse liver mitochondria are to be reported subsequently. In the case of MC, there was no instance of inhibition of glutamate oxidation in either normal or riboflavine-deficient mitochondria, which agrees with present knowledge that MC has not been shown to produce liver tumors in rats.

In conclusion, it may be said that the results of the present as well as those of a previous study (*11*) are compatible, for the most part in a qualitative way, with the idea that carcinogens may exert their effects *in vivo* by an initial inhibition of glutamate oxidation. A proper evaluation of the relationship between carcinogenicity and inhibition of glutamate oxidation, however, must await further investigations on a larger number and variety of carcinogenic and noncarcinogenic compounds. Furthermore, especially with the aminoazo dyes, which are extensively metabolized *in vivo*, such an appraisal must take into account the extent of modifying reactions that may play a decisive role in determining whether a substance can act as a carcinogen. There is little suggestion in the present work that an intermediate rather than the administered compound is the active carcinogen. The possibility that inhibition of glutamate oxidation, specifically, may be related to the carcinogenic process as

[7] Although a great deal is known about the metabolism of aminoazo dyes, the relative rates of their destruction *in vivo* is not known with any degree of certainty. Kensler *et al.* (*25*) found that liver slices in Krebs-Ringer solution fortified with glucose destroyed PAA at a somewhat faster rate than DPAA. Mueller and Miller (*26*) have indicated that the principal route of dye destruction (DPAA) is through reductive cleavage which appears to be catalyzed by at least two and probably still other flavoprotein enzymes. One of these studied by Mueller and Miller (*27*) parallels in properties and intracellular location, the TPN-cytochrome *c* reductase of the small granule fraction (*28*). In this system there appeared to be no correlation between carcinogenicity of various aminoazo dyes and their rate of reduction (*20*). The xanthine oxidase system of liver represents another flavoprotein enzyme which is active in the reduction of various nitrogenous groups including the azo linkage. In the xanthine oxidase-DPNH system, DPAA was very slowly reduced (*29*). In other unpublished experiments, PAA was rapidly reduced while DTAA and TAT were only very slowly reduced. A similar relationship was seen by Ross and Warwick (*30*) in the rate of reduction of various cytotoxic aminoazo dyes in the xanthine oxidase-xanthine system.

suggested in these studies is particularly intriguing because of the unique metabolic position of glutamate, at the crossroads between carbohydrate and protein metabolism. It is not inconceivable that impairment of glutamate oxidation, which is not on the direct path of the citric acid cycle, may represent a kind of metabolic derangement to which the cell can readily adjust by diverting glutamate into pathways of synthesis rather than oxidation in the attempt to establish a new steady state.

Summary

The effect of some carcinogens and noncarcinogens, especially the aminoazo compounds, on glutamate oxidation was studied in riboflavine-deficient and normal mitochondria of rat liver. It was found that in riboflavine deficiency, mitochondria were susceptible to the inhibitory action of the carcinogens DPAA, DTAA, TAT, 2FdiAA and CCl_4. In addition, the noncarcinogens PAA and DPAT were also inhibitory. ATAT and MC were not inhibitory. The P/O ratios in the presence of inhibitor in every case but CCl_4 remained constant, which suggested that the inhibitors were acting primarily on the oxidation process. The inhibition pattern of various substrate oxidations catalyzed by mitochondria was determined for some carcinogenic and noncarcinogenic aminozao compounds. The patterns were similar except in the case of the toxic noncarcinogen DPAT, in which there appeared a significant inhibition of succinate oxidation. The relation of the observed inhibitory effects on glutamate oxidation to the carcinogenic action of these compounds in animals is discussed.

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Some Epidemiological Aspects of Leukemia in Children [1]

MIRIAM D. MANNING, *M.D., M.P.H.*,[2] *and* BENJAMIN E. CARROLL, *M.A.*,[3] *Children's Cancer Research Foundation, Boston, Massachusetts, and National Cancer Institute,*[4] *Bethesda, Maryland*

There is no doubt that the recorded number of cases of leukemia has increased both in the United States, as reported by Sacks and Seeman (*1*), and in some European areas, as reported by Hewitt (*2*). The increase in the United States appears to be greatest in age groups over 50, according to Cook's study (*3*), but there is an increase in all age groups. Shimkin (*4*) suggests that limitations of death-certificate data (variation between incidence and mortality in a practically 100% fatal disease, and changes in classification in the population plus better diagnosis) may distort the figures. It would seem, however, that leukemia is underreported as a cause of death and that in spite of some artifacts, the increase of leukemia is real.

In April, 1953, Dr. Sidney Farber and Dr. Wilhelm Hueper (*5*) discussed the problem of environmental cancer and of potential uterine or transplacental exposure of the fetus to carcinogens that might enter the maternal organism before or during pregnancy. As a result of this discussion, it was suggested that effects on the fetus in mothers exposed to the following groups of agents be studied: estrogens, steroids, aromatic amines, ionizing radiation, benzol, arsenicals, and weed killers. Kaplan (*6*) suggested that we seek beyond benzol and irradiation and look for possible inciting agents among any chemicals, drugs, or body reactions such as hypersensitivity, which are capable of causing severe injury to hematopoietic tissue. Therefore, it also seemed wise to obtain medical information on the mother and child.

During August and September, 1953, a question sheet was devised covering the previously mentioned substances and a short but pertinent medical and work history of the mother was included, together with certain information about the index child [5] and his siblings. Preliminary sheets used in pilot studies showed that some very desirable information was unobtainable because of the limits of human memory and knowledge.

[1] Received for publication June 18, 1957.
[2] Surgeon, U. S. Public Health Service.
[3] Statistician.
[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[5] A child with leukemia or other malignancy, or a child selected as a control, is referred to as the index child.

The final question sheet—the result of 3 trials—was reviewed by Dr. William Baum and Mr. William Haenszel, of the National Cancer Institute. In most cases the mother was interviewed early in the course of the child's disease—after the initial trauma of the diagnosis was over. A casual approach, minimizing the possibility that the mother was in any way responsible for the disease, was used in all cases to avoid weighting the answers of the mother, and all mothers were questioned by the same individual. The questions were explained to the control mothers as a survey to compare them with women of other unstated categories. All mothers were most cooperative and interested and there were no refusals in any group. A field trial showed that it was impossible to get complete or accurate information by mail.

From September, 1953 to May, 1956, 373 mothers, whose children ranged from newborn to age 14, were questioned. All women, except controls, were mothers of children who were regular patients at the Children's Cancer Research Foundation in Boston, Massachusetts. Controls were randomly selected, excluding poliomyelitis cases, from patients at the Orthopedic Clinic of the Children's Medical Center in Boston. All cases of leukemia were diagnosed by histologic examination of the bone marrow, and other malignancies were also diagnosed from biopsy material. The index children included in this study were classified as follows:

1) Acute leukemia (type unspecified)..... 188
2) Lymphomas (including Hodgkin's disease)..... 42
3) Other cancer..... 93
4) Controls..... 50

Some interesting facts became apparent in the first question sheets, so a preliminary analysis was made. The results must, of course, be considered tentative. A more thorough analysis will be made when a sufficiently large number of cases has been collected to support it. The environmental factors will need to be analyzed in relation to one another and to age. Account also must be taken of the effect of multiple comparisons on the probability of occurrence of chance associations.

Table 1 shows that the mean age of mothers at birth of index children is several years higher in the leukemic and control groups than in other cancer and lymphoma groups. These differences are statistically significant. The dispersion of ages about the means also show statistically significant differences. An adequate number of cases has not as yet been interviewed in all groups to justify comparison with a general population.

The data collected about birth order suggest that real differences may exist among certain groups. The leukemic group has nearly twice as high a percentage of cases in birth order 3 as the other cancer group, for example, and considerable differences are seen among the groups in birth orders 4 and higher. These differences as yet are only suggestive, however, and a complete analysis of this characteristic must await the collection of a larger number of cases (table 2).

A question on smoking was included as a device for reliability. The

TABLE 1.—*Age of mother at birth of index child*

Age of mother	Leukemia	Lymphoma	Other cancer	Control
14–15	—	—	2	—
16–17	2	2	9	—
18–19	4	10	14	2
20–21	13	8	27	6
22–23	16	9	15	4
24–25	30	5	12	7
26–27	23	6	8	6
28–29	13	2	4	8
30–31	26	—	1	3
32–33	20	—	—	3
34–35	17	—	—	2
36–37	8	—	—	1
38–39	9	—	—	1
40–41	4	—	1	1
42–43	2	—	—	—
No. cases at all ages	187	42	93	44
Mean age of mother	29. 0	22. 5	22. 2	27. 4

percentage of mothers who smoked was found to be remarkably similar in all groups. Thirty-nine percent of the mothers of leukemic children smoked 10 or more cigarettes a day, as compared with 31 percent of mothers of children with lymphoma, 37 percent of mothers of children with other malignancies, and 38 percent of the control mothers.

Patient groups were higher than controls in both industrial and home exposures to possible carcinogenics, such as benzol, insect sprays, and floor and furniture wax. More cases will be needed to make statistically valid comparisons.

The widespread use of antibiotics by all groups was noted, all having about the same percentage of users. The chief suggestion of a real difference in this table is the higher percentage using "cold" tablets (usually antihistamines) in the leukemic group (table 3).

There is a significantly increased proportion of mothers with a history of hay fever, asthma, or hives in the leukemic and lymphomatous groups, as compared with those in the other cancer and control groups (table 4).

The children with leukemia and lymphoma also showed a significantly larger number with a history of allergy, as compared with children in

TABLE 2.—*Birth order of index child—percentage of total in each group*

Birth order	Leukemia	Lymphoma	Other cancer	Control
1	36. 7	35. 6	39. 7	43. 2
2	25. 5	31. 0	33. 3	34. 1
3	19. 7	16. 7	10. 8	13. 6
4	10. 1	11. 9	6. 5	2. 3
5	4. 8	2. 4	3. 2	2. 3
6 or higher	3. 2	2. 4	6. 5	4. 5
Total	100. 0	100. 0	100. 0	100. 0
Total number	188	42	93	44

TABLE 3.—*Mothers using certain drugs and antibiotics—percentage of total in each group*

Drug	Leukemia	Lymphoma	Other cancer	Control
Antibiotics	71. 8	69. 0	73. 1	72. 7
Sulfonamides	29. 8	40. 5	34. 4	34. 1
Aspirin	39. 9	33. 3	31. 2	22. 7
"Cold" tablets	21. 8	11. 9	10. 8	9. 1
Other drugs	19. 1	23. 8	23. 7	15. 9
Any listed drug	89. 4	83. 3	86. 0	77. 3
No listed drug	10. 6	16. 7	14. 0	22. 7
Total number	188	42	93	44

TABLE 4.—*Mothers having allergies—percentage of total in each group*

Allergy	Leukemia	Lymphoma	Other cancer	Control
Hay fever	15. 4	9. 5	5. 4	8. 0
Asthma	3. 7	2. 4	1. 1	6. 0
Hives	21. 8	21. 4	8. 6	10. 0
Other	0. 5	0	0	0
Any allergy	32. 4	31. 0	11. 8	20. 0
No allergy	67. 6	69. 0	88. 2	80. 0
Total number	188	42	93	50

TABLE 5.—*Presence of allergy in index child*

Group	Number of children	Percent with allergy
Leukemia	168	25. 0
Lymphoma	42	23. 8
Total, 2 groups	210	24. 8
Other cancer	93	10. 8
Control	50	12. 0
Total, 2 groups	143	11. 2
Total, all groups	353	

other cancer and control groups. A common manifestation of allergy in the children was eczema (table 5).

A cross tabulation of the relation between allergies in mothers and children gives a low coincidence of allergies in both mother and child in the leukemic group. This might indicate that the child of an allergic

mother starts life with a greater susceptibility, but not necessarily the same form of allergy as the mother, to sensitizing agents that may later depress the bone marrow (table 6).

TABLE 6.—*Relation between allergies in mother and index child—percentage of total in each group*

Allergy present	Leukemia	Lymphoma	Other cancer	Control
In mother and child	9.6	19.0	2.2	8.0
In mother only	23.4	11.9	9.7	12.0
In child only	12.2	9.5	6.4	4.0
In neither	54.8	59.6	81.7	76.0
Total	100.0	100.0	100.0	100.0

Almost twice as many mothers of children with leukemia had been exposed to therapeutic irradiation as mothers of children in the other cancer and control groups (19.5% as contrasted to 11.9%) (table 7). This question concerned any and all therapeutic irradiation prior to or during pregnancy with the index child without relation to site or dose.

TABLE 7.—*Radiation therapy of mother prior to or during pregnancy with index child*

Group	Number in group	Percent with radiation therapy
Leukemia	188	19.5
Lymphoma	42	10.7
Other cancer	93	12.2
Control	50	11.9

Of the 36 mothers who had received irradiation, the indications were as follows: 26 received therapy for dermatological conditions, usually acne; 8 received therapy for orthopedic conditions, usually bursitis or back pain; 2 received therapy for severe sinusitis.

It is impossible to estimate the dose of radiation received by the mother and it is improbable that the uterus received a large dose in any of the cases. Both exposure to X ray and allergy are being correlated with age and these data will be reported when more cases have been accumulated. Of the mothers of leukemic children with a positive history of allergy, 27 percent had received therapeutic irradiation.

Discussion

Relation to Hypersensitivity

Witts (*7*), Hueper (*8*), and others have suggested the possible influence of sensitivity in the etiology of leukemia. Vaughn (*9*) and Osgood (*10*) are among those who also have speculated on the subject. Law (*11*) states that too little attention has been given to chronic repeated exposures

to leukemogenic agents, consideration of the time-dose relationship, and the possible role of procarcinogens. Any agent that suppresses bone marrow might prove to be leukemogenic. Dreyfus and Bessis (*12*) found a classic leukemia developing in 6 of 17 cases of bone-marrow aplasia.

The data presented here are the first evidence that there may be a relationship between exposure to bone-marrow depressing agents, either in the uterus or postnatally, and the development of leukemia in hypersensitive children. That hypersensitivity may be transmitted in the uterus has been reported by Ratner *et al.* (*13*) as well as by others. It may be that the child receives from the mother a hypersensitive state, which may require later exposure to bone-marrow depressing agents in order to elicit the response of leukemia.

The finding that an increased number of mothers and children had a positive history of hay fever, asthma, or hives in the leukemic group may, if borne out in other studies, give significant clues to the cause of leukemia in children.

Relation to Ionizing Radiation

The relationship of ionizing radiation to leukemia has been reported by Furth (*14*), Warren (*15*), Kaplan (*16*), and others. Peller and Pick (*17*) have reported the increased incidence of leukemia among radiologists. Brown and Abbatt (*18*) have suggestive evidence that observed deaths from leukemia among patients who had received irradiation for ankylosing spondylitis were at least 5 and probably 10 times greater than the expected number of such deaths. Clark (*19*) has associated carcinoma of the thyroid in children with previous irradiation to the neck and chest areas. Simpson, Hempelman, and Fuller (*20*), in a series of 1,722 children who had received X-ray therapy to the thymus gland, found 7 cases of leukemia and 6 cases of carcinoma of the thyroid in the 1,400 whom they could follow, a significantly higher incidence than was found among untreated siblings, or in a general population. In the children previously receiving X ray, the observed rate of leukemia was 7 compared with an expected rate of 0.6, and the observed rate of carcinoma of the thyroid was 6 compared with an expected rate of 0.08. In contrast no cases of leukemia or cancer of the thyroid occurred in the untreated group, whose expected rates were the same as those of the treated group.

Stewart (*21*) has presented data showing a significantly higher incidence of pelvimetry by X ray in mothers of children with malignancies, including leukemia, than in mothers of a control group. In reporting 547 fatalities from leukemia or other malignancies in children under 10 years of age, she found that 42 of the mothers of 269 leukemic fatalities had diagnostic radiation of the abdomen antenatally, compared to only 24 mothers of controls. Other malignant diseases accounted for 278 cases, and 43 of these mothers had diagnostic abdominal X rays as compared to only 21 mothers of the controls. Her data was obtained by questionnaire. The present study was started before Dr. Stewart's findings were reported and since pelvimetry was not included as a specific question

all of our data cannot be compared with hers. It is interesting to note, however, that in the present study the increased exposure to therapeutic irradiation was found only among mothers in the leukemic group and was not common to both groups of malignancies as Dr. Stewart found.

Conclusions

The increased incidence of allergy in mothers and children in the leukemic group and the greater number of mothers of leukemic children who had been exposed to therapeutic X ray are the most significant findings in this study to date. This evidence suggests that leukemia in children may be the result of exposure of a susceptible individual to bone-marrow depressing agents.

A larger number of cases in all groups is necessary and data should be obtained that will be comparable to material in other studies. We believe that the data presented justify and should stimulate further investigation along this line of approach to the problem of acute leukemia in children.

Summary

1) Mothers of children with acute leukemia were questioned by the same individual, and mothers of children who had other malignancies or were orthopedic cases, excluding cases of poliomyelitis, served as controls. A group of lymphoma cases was separated from the leukemic group.

2) No correlation was found with smoking habits of mothers and the incidence of leukemia, lymphoma, or the other malignancies of childhood.

3) A significantly larger number of mothers of children in the leukemic and lymphomatous groups had a positive history of hay fever, asthma, or hives compared with the other malignancy and control groups.

4) A higher percentage of mothers of the leukemic children used "cold" tablets (usually antihistamines) compared with the other groups.

5) There was a significantly higher incidence of allergic manifestations in the leukemic children compared with other groups.

6) Almost twice as many mothers of leukemic children had received therapeutic irradiation prior to the birth of the index child compared with mothers in the other groups.

7) The possible relationship between leukemia, irradiation, bone-marrow depressing agents, and hypersensitivity is discussed briefly.

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Embryonic Inductive Action of Normal and Leukemic Bone Marrow of the Rat. II.[1, 2]

SULO TOIVONEN *and* LAURI SAXÉN, *Zoological Laboratory and Department of Pathology, Section II, University of Helsinki, Helsinki, Finland*

In a series of classical experiments performed in the 1920's, Spemann and his school (*1*) showed that certain parts of the embryo possess the capacity to regulate the differentiation of other parts, and subsequent investigations hinted very strongly at the involvement of specific chemical factors, or inductors (*2*). Later, Holtfreter (*3*) found that this inductive capacity is not confined to embryonic tissues. He showed that adult tissues, too, are capable of induction and thus apparently contain certain active factors. The next step was the observation that the inductive action of adult tissues is not of a random nature because the tissues have specific influences (*4–7*). These discoveries naturally raise the question of the function of these factors in adult tissues. Is their action on embryonic tissues a purely haphazard secondary effect or do the same, or similar factors, regulate both embryonic differentiation and subsequent growth and regeneration of the tissues (*8*, *9*)? From the assumption that the answer to the latter question is in the affirmative, the working hypothesis for the present study was deduced: If tissue growth and differentiation are always regulated by specific factors, it seems logical to assume that, owing to these factors, the situation will differ from the normal in malignant, markedly abnormal growth, and that the differences will be detectable by tests with embryonic, totipotent undifferentiated tissue.

Normal and leukemic bone marrow was used in preliminary experiments (*10*) in which it was found that leukemic tissue had lost its inductive capacity, thus differing from all normal tissues investigated. This observation was made, however, on scanty material, and the tissue investigated was derived from one rat only. The present results were obtained by the same technique and with the same tissues but with more ample material.

Materials and Methods

The bone marrow used for implantation was derived from young Wistar rats: 4 leukemic and 4 normal.

Seven-day-old Wistar rats were injected subcutaneously with 0.1 cc.

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of a homogenate prepared from leukemic tumor tissue diluted with the same volume of physiological saline. Leukemia had been induced by gastric instillation of methylcholanthrene, which resulted in the development of typical, transplantable myeloid leukemia (*11*, *12*).

Following transplantation, the peripheral-blood picture of the animals was continuously observed. After the manifestation of leukemia, the rats were killed and their tissues used for implantation. The peripheral-blood picture of the 4 rats at the time of sacrifice is given in table 1.

TABLE 1.—*Peripheral-blood picture of 4 leukemic rats at time of sacrifice**

	Leukemic rats				Normal values†
	1	2	3	4	
Leukocytes	97, 000	44, 400	89, 300	48, 300	6, 000–25, 000
Granulocytes	15	25	42	12	19–40
Myelocytes	9	8	17	38	‡
Lymphocytes	57	58	36	44	62–75
Monocytes	19	9	5	6	1–6
Normoblasts (per 100 leukocytes)	11	2	2	4	0–3§

*All data: Wasastjerna, personal communication.

†The normal blood values are indicated as suggested by Schermer (*13*). They were found to correspond to the values obtained in the Wistar strain in our laboratory.

‡In investigations carried out over several years in our laboratory, myelocytes have not been observed in the peripheral blood of normal rats.

§In our laboratory, normoblasts from 0 to 3 per 100 leukocytes have been observed in young rats.

The age of the leukemic rats varied between 8 and 10 weeks. Only rat #4 of this group was clinically in poor condition at the time of sacrifice. Specimens were taken for histological examination from animals #1, #3, and #4. The organs of #2 were not histologically investigated owing to a technical mistake. In the *bone-marrow* specimens, over 90 percent of the cells were granulated and similar in type, displaying a large nucleus and a clearly visible nucleolus. These cells mostly resembled atypical promyelocytes. In addition, some plasma cells, occasional normoblasts, and a few normal granulocytes were observed (fig. 1). The *liver* and the *spleen* exhibited abundant infiltrations of myeloid cells.

The age of the 4 normal controls varied between 9 and 11 weeks. Nothing deviating from the normal was observed in their peripheral-blood picture, or histologically.

Immediately after sacrifice the entire bone marrow was taken from both femurs. The pieces were minced and fixed in 70 percent alcohol and used for implantation 8 to 48 hours later. Before implantation, the pieces were washed for 3 to 4 hours in Holtfreter-Ringer solution, which was repeatedly changed.

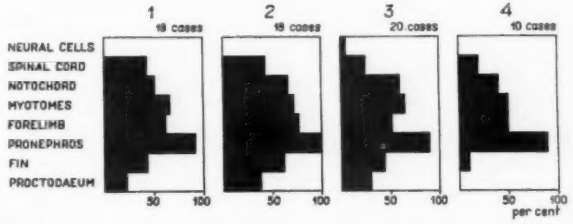
The tissues to be investigated were implanted into the blastoceles of young gastrulae of *Triturus vulgaris*. After the operation, the embryos were reared for 10 to 14 days in a sterile Holtfreter-Ringer solution to which sulfapyrimidine (Elkosin,® Ciba) had been added. The animals were fixed in Bouin's solution, embedded in paraffin, and serially sectioned.

Because the object was to compare the inductive activity in 2 parallel series, particular care was taken to avoid any differences in treatment. Hence, not only were operation and rearing performed by exactly the same technique but also the implantation material was consistently treated in the same way and for the same length of time. On inspection of the preparations, the same criteria were used for incorporation in the series or for discarding. Besides a minimum age of 10 days, the conditions for incorporation were that the inductor should be observable on histological examination and that it should be in contact with the reactive epidermis. Numerous larvae were discarded because the implant was either embedded in the entoderm or absent. No additional differentiations were found in these cases.

Results

Normal Bone Marrow

In this series a total of 148 gastrulae were operated on, and 103 lived long enough to be examined microscopically. From these, 24 were discarded because no implant was found and 13 because the implant was embedded in the entoderm. Thus, 66 were approved. The previously published results (*10*) are included in table 2, so that the series comprises a total of 100. There were only 2 larvae in which no induction was observed, although the implant was in contact with the ectoderm; thus 98 percent of them were clearly positive. Just as in previous experiments with guinea-pig bone marrow (*14–16*), the reaction was mainly mesodermal. No cephalic structures or sensory organs were encountered, but in about one third a short spinal-cord primordium was seen at the tip of the induced tail. Furthermore, in some larvae dispersed neural cells or aggregations of cells were observed that could not be classified exactly. The remainder of the induced structures were mesodermal—a secondary pronephros being the commonest. In text-figure 1, the inductive influence of the bone marrow of each of the 4 normal rats is shown, and in figures 2 and 3 some typical instances of induced structures are depicted.



TEXT-FIGURE 1.—Inductive capacity of the 4 normal bone-marrow tissues examined.

Leukemic Bone Marrow

A total of 259 implantations were performed, and 186 larvae lived long enough to be examined microscopically. In 57 the implant was not found, and in 21 the implant was embedded in the entoderm of the host. Thus,

TABLE 2.—*Inductions caused by normal and leukemic rat bone marrow observed on histological examination**

Implantation tissue		No. of larvae	No. of positive larvae	Induction percentage	Structures induced										
					Forebrain	Eye	Nose	Neural cells	Spinal cord	Notochord	Myotomes	Pronephros	Forelimb rudiment	Fin	Proctodeum
Leukemic bone marrow															
1955		33	0	0											
1956	1.	12	0	0											
	2.	12	6	50	—	—	—	—	3	4	4	5	5	4	—
	3.	50	1	2	1	—	1								
	4.	34	2	6	—	1	—	1	—	—	—	1	—	—	1
Total		141	9	6	1	1	1	1	3	4	4	6	5	4	1
Normal bone marrow															
1955		34	34	100	—	—	—	3	8	19	28	31	15	26	22
1956	1.	18	18	100	—	—	—	—	8	10	12	17	11	8	4
	2.	18	18	100	—	—	—	—	8	12	13	18	14	11	7
	3.	20	19	95	—	—	—	1	5	12	13	18	10	9	6
	4.	10	9	90	—	—	—	—	2	4	5	9	5	1	
Total		100	98	98	—	—	—	4	31	57	71	93	55	55	39
Simultaneous implantation.....		22	22	100	—	—	—	—	8	14	19	19	8	11	10

*The table also includes previously published results of experiments performed in 1955 (*10*).

108 were approved. In addition, 33 previous larvae are included in the tables. Out of these, 132 (94%) were negative inasmuch as no secondary structures had developed, although a varying degree of epidermal proliferation was observable in one third of them. In 6 of the 9 positive larvae, bone marrow from the same leukemic rat had been used. All of these exactly resembled the normal controls, while 6 other operations performed with bone marrow from the same rat were completely negative. Of the remainder of the positive larvae, 2 were definitely atypical bone-marrow inductions, the reaction being weakly archencephalic. In the third, in which bone marrow from rat #4 had been used, a very weak mesodermal reaction (pronephros + proctodeum) occurred. In figures 4 and 5, two inactive bone-marrow implants are shown. In 1 of these, the above-mentioned epidermal proliferation is seen without any additional, differentiated structures.

In addition, a limited experimental series was made in which bone marrow from a normal and from a leukemic animal was simultaneously implanted into the same blastocele (normal no. 4 + leukemic no. 4). The resulting inductions (table 2) corresponded both qualitatively and quantitatively to the results obtained with normal bone marrow. (Only larvae in which both implants were histologically observable were approved.)

Discussion

In the present experiments, just as in previous experiments with bone marrow, normal rat bone marrow proved to be an almost purely mesodermal inductor (*14–16*). In regard to quality and intensity of the inductive effect, the bone marrow of different rats was very similar (text-fig. 1).

The leukemic bone marrow was derived from 5 rats with the disease, which had been established from the peripheral-blood picture. The bone marrow of 4 of these animals proved to be almost completely devoid of inductive capacity, which is very unusual since no adult tissue has hitherto been found to be entirely inactive. In contrast, the bone marrow of 1 leukemic rat gave a typical, mesodermal bone-marrow reaction in half the experiments, while in the other half it was completely inactive. The explanation of this seems to be that the leukemia of this rat was in an earlier stage than that of the other animals, and, therefore, the bone marrow employed was not yet completely infiltrated by leukemic tissue. Thus, the implants must also have contained intact tissue. In this rat the peripheral-blood picture was more benign, and the leukocyte count and the number of juvenile forms were lower than in the others.

With regard to the difference between the normal and the leukemic bone marrow, the first point to be discussed is the extent to which normal bone marrow may be regarded as adequate control material for leukemic tissue. The leukemia was induced by Shay *et al.* (*11*, *12*) by gastric installation of carcinogens. These investigators emphasized that the leukemia in question is typical, being characterized by a positive blood picture and bone-marrow structure, and tissue infiltrations. From this investigation, and from our histological examinations, it appears that the bone marrow was

neoplastic. Although rats of the same age and strain were used both as experimental animals and as controls, the transplantable neoplastic tissue employed in the experiments was originally derived from other animals and was in a way much "older." Hence, the comparability of the tissues employed may, perhaps, be regarded as controversial, but the fact that normal *and* malignant bone-marrow implants were used is beyond doubt.

The second question is whether the difference in the results can be attributed to technical factors. In the description of the method it was emphasized that *Triturus* gastrulae were used in both groups and that all procedures were exactly the same. For instance, the alcohol treatment of the tissues to be implanted varied within the same limits, since prolonged treatment may reduce the inductive capacity of a tissue. The experiments were performed during the period from May to July, 1956, normal and leukemic bone marrow being used alternately. Hence, neither time nor laboratory temperature could have influenced the results to any material degree. Furthermore, experiments performed in 1955 yielded similar results (*10*). Thus, no technical cause seems to be responsible for the absence of inductive activity of the leukemic bone marrow.

Third, it may, of course, be suggested that the leukemic bone marrow contains some factor, absent in normal bone marrow, that inhibits the influence of the mesodermal inductive agents. In order to eliminate this possibility, a series was made in which normal and leukemic bone marrow were simultaneously implanted into the same gastrula. Exactly the same kind of inductions resulted as in the series made with normal bone marrow. Hence, it seems obvious that *in leukemic bone marrow one or more agents are lacking or inactivated which are always present in normal bone marrow and which, after implantation into a young gastrula, bring about the formation of additional mesodermal structures from the ectoderm.*

For many reasons, the present observation cannot as yet be coupled, even in part, with the problem of carcinogenesis. First, the finding in question cannot be generalized so as to refer to other malignant tissues, or even to other leukemias, and, owing to the time-consuming nature of the technique employed, the exploration of other tissues is a distant goal. Second, information regarding both the process of embryonic induction and carcinogenesis is so scanty that correlation of the relevant data is impossible. Purely as a working hypothesis this correlation is tempting, however. As mentioned in the introduction, it seems very probable that embryonic differentiation is regulated by certain active factors (*2*), and numerous investigations have, in addition, yielded evidence to suggest that this chemical regulation is not confined to early gastrulation alone, later organogenesis being also, at least in part, subject to the regulation of such substances (*17*). If, for instance, embryonic blood formation is regulated by specific factors, it seems reasonable to assume that the hematopoiesis of adult individuals is similarly regulated. On this assumption, the proper interpretation of the present result would seem to be that the factor regulating normal leukopoiesis is destroyed or absent

in myeloid leukemia, and that differentiation is consequently disturbed. Disturbed differentiation may, moreover, be the cause of decreased growth inhibition, as might be expected, for instance, on the basis of Osgood's (*18*) theory. The fact that, in the present experiments, the differentiating principle that is normally present in the bone marrow was not capable of bringing about differentiation of totipotent ectoderm directly into blood cells does not constitute evidence against the view that highly specific factors are involved. The explanation may be that the undifferentiated ectoderm of the young gastrula reacts to the highly specific inductors of adult rats only in a manner typical of its age, *i.e.*, by forming primary mesodermal structures. According to this interpretation, the small number of different, induced structures observed in implantation experiments is due not so much to scantiness in the repertoire of inductors but to the limited reactive capacity of the ectoderm.

On the basis of the working hypothesis advanced here, it is the authors' purpose to continue the investigation of the inductive action of malignant tissues. Hitherto it has only been established that a certain malignant tissue, leukemic rat bone marrow, is not capable, as are all normal tissues, of bringing about differentiation of embryonic, totipotent ectoderm.

Summary

The embryonic inductive capacity of normal and leukemic rat bone marrow was investigated in implantation experiments with *Triturus* gastrulae. With the bone marrow obtained from 5 normal and 5 leukemic rats, 100 control operations and 141 experimental operations were performed. The normal bone marrow proved to be a strong, almost purely mesodermal inductor. The bone marrow of the 4 leukemic rats investigated was practically devoid of inductive action—only 3 instances of weak, untypical induced structures being encountered in a total of 129 larvae. The bone marrow of the fifth leukemic rat differed from that of the others inasmuch as it yielded a definite reaction in half the experiments while the remainder were negative. When normal and leukemic tissue were simultaneously implanted, the result was the same as in the experiments with normal bone marrow alone. From these results the conclusion is drawn that the factor, present in normal bone marrow, which induces mesodermal structures is inhibited or absent in leukemic bone marrow.

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PLATE 89

FIGURE 1.—Photomicrograph of leukemic bone marrow used for implantation. Hematoxylin and eosin. × 1,000

FIGURE 2.—Photomicrograph of typical induced structures obtained with normal bone marrow. Ventrally of the intestine is a marked, secondary accumulation of myotomes with a notochord in the center. *Above* and to *left* lies a pronephric tubule. Between intestine and induced structures, remnants of the implant are seen. Hematoxylin and eosin. × 100

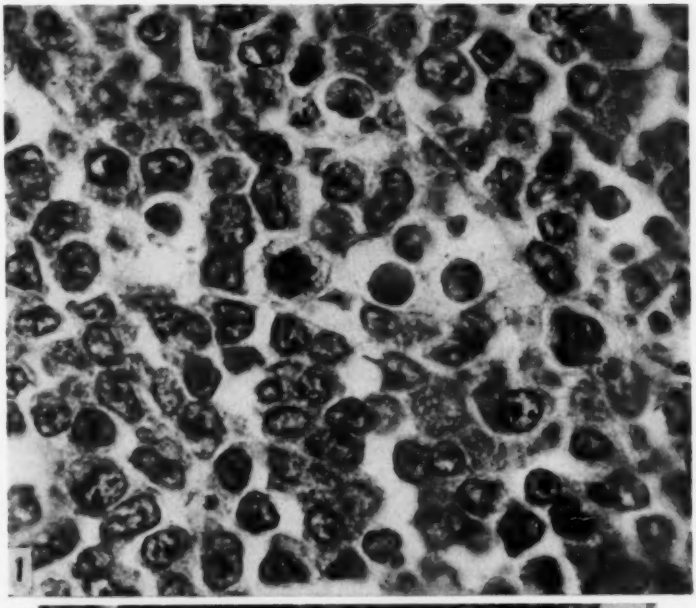

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PLATE 90

FIGURE 3.—General view of animal treated with normal bone marrow. The ventral epidermis has developed a secondary tail, in the anterior portion of which is the primordium of an additional limb.

FIGURE 4.—Photomicrograph of a 13-day-old tadpole, in which a leukemic bone-marrow implant is seen in contact with the ectoderm. No reaction is observable. Hematoxylin and eosin. × 100

FIGURE 5.—Photomicrograph of a leukemic implant between the intestine and ventral ectoderm of a 14-day-old tadpole. In the ectoderm slight proliferation is obervable, but no differentiation. Hematoxylin and eosin. × 100

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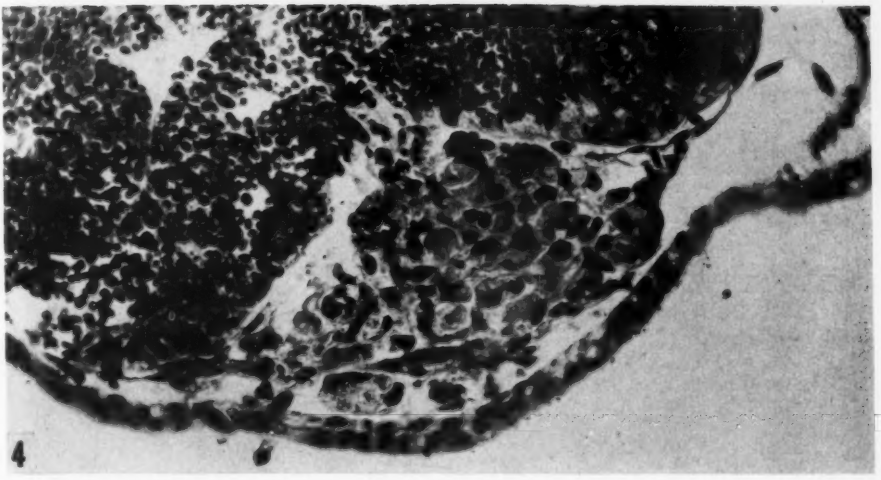
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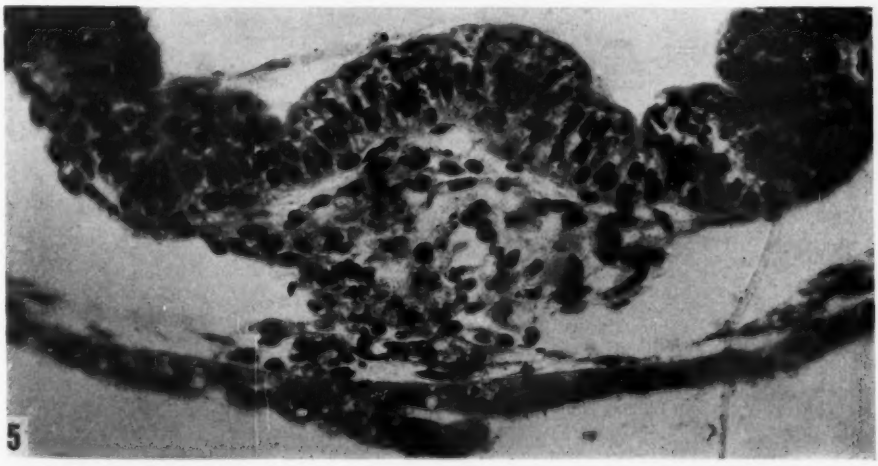
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An Interpretation of Survival Rates: Cancer of the Breast [1]

SIDNEY J. CUTLER,[2] MATTHEW H. GRISWOLD, *M.D.*,[3] *and* HENRY EISENBERG, *M.D.*,[3] *National Cancer Institute, Bethesda, Maryland, and Connecticut State Department of Health, Hartford, Connecticut*

Survival and "cure" rates provide an objective measure for evaluating the effectiveness of cancer therapy. The *5-year* rate has been generally accepted as the index. Thus, the Joint Committee on Reporting Cancer End Results,[4] in issuing its recommendation for "A Standardized Method for Reporting Cancer End Results," oriented its discussion to the reporting of *5-year end results* (*1*).

The usefulness of the 5-year survival rate was amply illustrated in a recent monograph published by the Connecticut State Department of Health (*2*). The Connecticut experience, based on the follow-up of a total of 57,000 cancer patients, indicated that the percentage of patients who were alive 5 years after diagnosis increased from 25 percent for those diagnosed from 1935 through 1940 to 32 percent for those diagnosed from 1947 through 1951. The increase was both absolutely and relatively greater for patients with localized lesions than for patients with involvement of regional tissue. The magnitude of the improvement was impressive for cancers that were primary in the large intestine, rectum, prostate, uterus, and bladder, and rather small for cancers of the stomach and lung. Thus, the 5-year survival rate provided a means for comparing the effectiveness of cancer therapy with respect to calendar period, stage of disease at diagnosis, and primary site. The monograph also contains data showing the influence of sex, age, and method of treatment on survival.

Although the 5-year survival rate is a very useful index for measuring the influence of various factors on the survival of cancer patients, evaluation of survival at the end of the 5th year after diagnosis has no special virtue. For example, the comparisons mentioned with respect to calendar period, primary site, and stage of disease could have been based just as legitimately on 3-year or 7-year rates. The use of the 5-year rate is simply a convention and should not be considered equivalent to cure.

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[2] Head, Clinical Biometry Section, National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Chief and Internist, respectively, Division of Cancer and Other Chronic Diseases.

[4] The Joint Committee was made up of representatives of the American College of Surgeons, the College of American Pathologists, the American College of Radiology, the American Cancer Society, and the U. S. Public Health Service.

Examination of the survival experience of cancer patients beyond 5 years is therefore desirable in order to provide a basis for interpreting the significance of 5-year survival rates and to provide insight into the meaning of survival rates in general.

This discussion, which is intended as an illustration of a methodological approach, will be limited to the survival experience of females with cancer of the breast. Since prognosis varies sharply with the primary site of the tumor, a significant analysis of survival among cancer patients must deal with specific sites. The survival outlook for patients with cancer of the breast is relatively favorable and provides an opportunity for study of survival after 5 years. Breast cancer accounts for about one fourth of all cancer in females, providing a large set of data for analyses—9,121 breast-cancer cases were diagnosed in Connecticut hospitals during the period 1935–53. The number of breast-cancer cases classified by stage of the disease and age of patient at diagnosis is given in table 1.

TABLE 1.—*Number of breast-cancer cases diagnosed in Connecticut hospitals, 1935–53, by age and stage of disease at diagnosis*

Stage of disease at diagnosis	Age at diagnosis						
	Total	Under 45	45–54	55–64	65–74	75 and over	Unspecified
All stages	9121	1925	2172	2251	1790	918	65
Localized	3849	869	851	909	764	431	25
Regional involvement	4050	841	1068	1041	757	318	25
Remote metastases	730	113	145	194	180	97	1
Unspecified stage	492	102	108	107	89	72	14

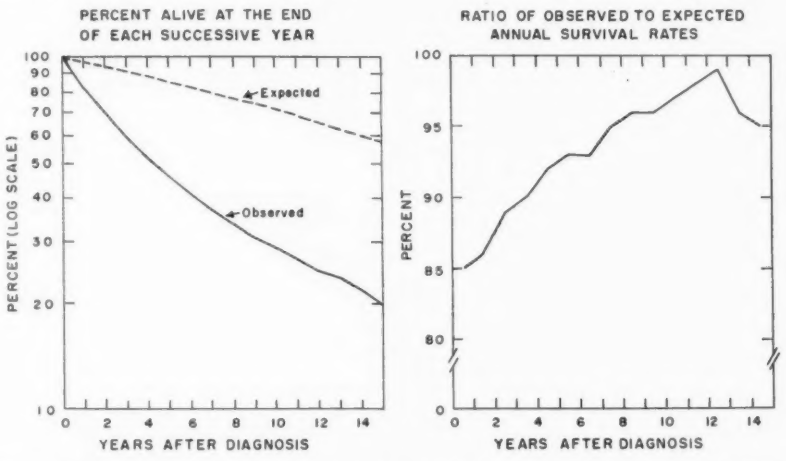
The changing rate at which the cohort of breast-cancer patients was depleted over a period of 15 years is depicted on the left side of text-figure 1.[5] The logarithmic scale used for plotting the percentage of survivors at the end of successive intervals permits visual evaluation of the rate of depletion (mortality) during successive intervals. A steep slope indicates a high mortality rate; a gradual slope indicates a relatively low mortality rate. This chart reveals that the mortality rate among breast-cancer patients tapers off the first few years after diagnosis.

To provide a frame of reference, the expected survival experience is indicated for a group of women from the general population, with an age distribution similar to that of the breast-cancer cases. The expected survival percentages were taken from the Connecticut Life Table for white females, 1939–41. The chart (*see* left side) reveals that the observed survival curve tends to become parallel to the curve for the general population. This means that after a number of years mortality among

[5] Standard actuarial procedures were used in computing the survival rates. The methodology is explained in the basic monograph (*8*).

the cancer patients under study approached the level observed in the general population.[6] Since elimination of the excess mortality risk due to cancer is a fundamental goal of cancer therapy, it is of interest to determine: 1) At what point, if any, does mortality for the surviving members of a patient cohort become essentially the same as in the general population, and 2) what proportion of patients are alive at that point.

An arithmetic measure of the relationship between the survival pattern of a patient cohort with that of the general population is provided by the ratio of the survival rates for the two populations over specified periods of time. Thus, in table 2, a comparison is made for 15 successive annual intervals of the observed survival rates for women with cancer of the breast with the corresponding rates for the general population (expected rates).[7] A ratio of 100 percent indicates that the observed survival



TEXT-FIGURE 1.—Observed and expected survival rates for breast-cancer cases diagnosed in Connecticut hospitals, 1935–53.

rate for a 1-year interval was equal to the expected rate. For convenience the ratio of observed to expected annual survival rates will be referred to as the *annual survival ratio*. These ratios are depicted in graphic form on the right side of text-figure 1. Examination of table 2 and text-figure 1 (*see* right side) reveals that for the cohort of breast-cancer patients the annual survival ratio was less than 100 percent throughout 15 years of follow-up. Although the annual survival rate increased during 13 successive years of follow-up, it remained below the level of the general population. During each of the 15 years, mortality in the cohort of breast-cancer patients was greater than expected on the basis of general population experience.

[6] Since the prevalence in the general population of any specific form of cancer is very low, the mortality experience of the total population of Connecticut is essentially equal to the mortality level for a cancer-free population, *i.e.*, free of the specific form of cancer in question.

[7] The general population survival rates are adjusted on the age distribution of the cancer patients.

To further illustrate that a 5-year survival is not equivalent to cure, let us consider the survival experience during the second 5-year interval after diagnosis. Forty-six percent of the original group of breast-cancer patients were alive 5 years after diagnosis. By the end of the tenth year, only 29 percent were still alive. However, if during the sixth through the tenth years after diagnosis, mortality had been reduced to the level observed in the general population, 38 percent of the original cohort would have been alive at the end of 10 years. In other words, 63 percent of

TABLE 2.—*Observed and expected interval survival rates for breast-cancer cases diagnosed in Connecticut hospitals, 1935–53*

Years after diagnosis	Interval survival rates (*per thousand*)		Annual survival ratios† (*percent*)
	Observed	Expected*	
0–1	829	972	85
1–2	833	971	86
2–3	859	969	89
3–4	868	967	90
4–5	892	966	92
5–6	900	964	93
6–7	899	964	93
7–8	916	962	95
8–9	920	961	96
9–10	927	961	96
10–11	929	959	97
11–12	940	958	98
12–13	941	953	99
13–14	915	953	96
14–15	906	949	95

*Based on Connecticut Life Tables for white females 1939–41.

†Ratio of observed to expected annual survival rates. The standard errors of the annual survival ratios for the first 12 years are 1.0 percent or less. The standard errors for the 13th, 14th, and 15th years are 1.2, 1.6, and 2.0 percent, respectively. Thus, additional data are needed to evaluate the apparent reversal in the trend of ratios beyond the 13th year.

the breast-cancer patients alive at the end of the fifth year lived through the end of the tenth year. On the basis of general population experience, 83 percent would have survived this interval.

One need not insist that the annual survival ratios reach unity (100%) before one is willing to conclude that mortality for the surviving members of a patient cohort has been reduced to an "acceptable" level. Even if treatment was successful and all traces of the tumor were in fact destroyed, it may be unduly optimistic to expect no reduction in life expect-

ancy in people undergoing a radical course of therapy.[8] Let us therefore determine what annual survival ratios of 99, 98 percent, etc., mean in terms of reduction in life expectancy and then consider how much of a reduction might be "acceptable."

In large part, we are concerned with the life expectancy of women who were between 45 and 65 years of age at diagnosis. This age range includes nearly half of all women with cancers of the breast. Table 3 indicates the effect on average life expectancy of annual survival ratios of 95 through 99 for women within this age range at diagnosis. For example, a group of women who were 45 at diagnosis, and survived 5 years, attained age 50. If their survival experience were "normal" from that point on, the average life expectancy for the group would be 24.2 years. A one percent reduction in the annual survival rates from age 50 on (annual survival ratio of 99) would decrease average life expectancy by 3.3 years, or 14 percent; a 2 percent reduction would decrease average life expectancy by 6.0 years, or 25 percent. If these women had survived 10 years, they would have attained age 55, with an average remaining life expectancy of 20.3 years. An annual survival ratio of 99 from that point on would reduce life expectancy by 12 percent; an annual survival ratio of 98 would reduce life expectancy by 22 percent; an annual survival ratio of 97 would reduce life expectancy by 29 percent, etc.

Comparison of the average life expectancy of various population segments provides a frame of reference for evaluating the magnitude of the

TABLE 3.—*Average remaining years of life for alternative annual survival ratios, white women at age 50 to 70*

	Beginning age				
	50	55	60	65	70
At annual survival ratio of 100*	24.2	20.3	16.8	13.4	10.4
At annual survival ratio of 99	20.9	17.9	15.1	12.3	9.7
Reduction: years	3.3	2.4	1.7	1.1	0.7
percent	(14)	(12)	(10)	(8)	(7)
At annual survival ratio of 98	18.2	15.8	13.6	11.3	9.0
Reduction: years	6.0	4.5	3.2	2.1	1.4
percent	(25)	(22)	(19)	(16)	(13)
At annual survival ratio of 97	16.3	14.4	12.5	10.5	8.6
Reduction: years	7.9	5.9	4.3	2.9	1.8
percent	(33)	(29)	(25)	(22)	(18)
At annual survival ratio of 96	14.6	13.1	11.5	9.8	8.1
Reduction: years	9.6	7.2	5.3	3.6	2.3
percent	(40)	(36)	(31)	(27)	(23)
At annual survival ratio of 95	12.8	11.7	10.4	9.0	7.5
Reduction: years	11.4	8.6	6.4	4.4	2.9
percent	(47)	(43)	(38)	(33)	(28)

*Taken from Connecticut Life Tables for white females, 1939–41.

[8] It should be noted, however, that preliminary data for patients with other forms of cancer, *e. g.*, uterus and large intestine, indicate that annual survival ratios of 100 percent can be achieved, and even may be exceeded.

alternative reductions in life expectancy given in table 3. For example, at age 50, the average life expectancy for white males is 23.0 years compared to 27.1 years for white females; a difference of 15 percent. Beginning at the same age, the life expectancy of nonwhite males is 24 percent less than that for white females (*3*). Comparison of populations in different geographic regions of the United States reveals that differences may be quite substantial. For example, for white females at age 60, the average life expectancy in the Middle Atlantic States is 17.7 years compared with 21.5 years in the West South Central States, a difference of 18 percent (*4*). Thus, different segments of the general population may differ by as much as 15 to 24 percent with respect to life expectancy. One might, therefore, take the position that achieving a reduction in life expectancy of no more than 25 percent is a reasonable therapeutic goal. For the "average case" of breast cancer (45–65 years of age), this corresponds to achieving a level of annual survival ratios of about 98 percent.

Annual survival ratios for 15 successive intervals are given in table 4, by age and stage at diagnosis. The corresponding cumulative percentage of survivors at the end of each interval (or beginning of the next interval) is given in table 5. The follow-up year in which an annual survival ratio of 98 was reached by each group of breast-cancer patients is indicated in table 6. For breast-cancer cases classified as localized at time of diagnosis, the arbitrarily selected "acceptable" level of continuing survival (a ratio of 98 or better) was reached in the 8th year after diagnosis; among regional cases this level wasn't reached until the 12th year. The "acceptable" level was reached by 56 percent of the

TABLE 4.—*Annual survival ratios, by stage and age at diagnosis: breast-cancer cases diagnosed in Connecticut hospitals, 1935–53*

Years after diagnosis	Age at diagnosis					
	All ages	Under 45	45–54	55–64	65–74	75 and over
	(Percent)					
All stages combined						
0–1	85	88	87	84	83	80
1–2	86	85	84	85	88	90
2–3	89	87	88	88	89	94
3–4	90	89	90	90	89	90
4–5	92	91	92	91	93	101
5–6	93	94	93	92	95	84
6–7	93	94	93	92	89	104
7–8	95	95	94	94	95	105
8–9	96	95	97	95	96	97
9–10	96	95	96	96	100	103
10–11	97	96	97	96	96	99
11–12	98	96	95	100	106	109
12–13	99	97	94	103	105	103
13–14	96	98	98	95	88	101
14–15	95	94	96	96	103	79

TABLE 4.—*Annual survival ratios, by stage and age at diagnosis: breast-cancer cases diagnosed in Connecticut hospitals, 1935–53*—Continued

Years after diagnosis	Age at diagnosis					
	All ages	Under 45	45–54	55–64	65–74	75 and over
	(Percent)					
Localized						
0–1	96	97	97	96	96	94
1–2	94	92	92	94	95	95
2–3	94	92	95	94	95	96
3–4	96	95	94	96	96	97
4–5	96	95	96	94	95	106
5–6	96	97	96	95	96	88
6–7	95	98	97	94	89	105
7–8	98	98	96	98	95	107
8–9	96	96	98	94	97	98
9–10	98	96	98	98	101	107
10–11	97	97	98	96	100	102
11–12	98	98	94	100	110	104
12–13	98	98	93	102	100	93
13–14	98	98	98	95	100	111
14–15	98	94	95	104	107	101
Regional						
0–1	85	86	87	84	84	79
1–2	80	79	77	80	82	85
2–3	83	82	83	81	83	95
3–4	84	83	85	83	82	83
4–5	89	87	90	87	92	93
5–6	91	91	90	90	95	78
6–7	90	90	88	90	90	110
7–8	93	93	92	88	97	103
8–9	95	94	95	97	94	92
9–10	94	92	94	94	99	87*
10–11	97	96	99	98	90	78
11–12	98	94	96	103	94	133
12–13	100†	92	96	106	115	135
13–14	92	96	97	93	58	69
14–15	91	92	98	87	78	0

*Additional data are needed to evaluate the apparent reversal in the trend of ratios beyond this interval. The observed reversal may have occurred by chance, due to sampling variation. The standard errors of the ratios for the last 5 intervals (all ages combined) are 1.7, 1.8, 1.8, 3.3, and 4.1 percent.

†The annual survival ratios beyond this interval are based on less than 10 survivors and are subject to considerable sampling variation.

localized cases and by only 19 percent of the regional cases. The less favorable survival experience of the regional cases is further emphasized by the fact that within the 15-year period of follow-up the annual survival ratios did not attain a level of 98 or better in 3 of the 5 age groups.

The data suggest a reversal after the 13th year in the trend of annual survival ratios for regional cases (table 4). This apparent reversal may be due to long-term recurrence of breast cancer. Additional data are needed

TABLE 5.—*Percentage of cases alive at end of each annual interval, by stage and age at diagnosis: breast-cancer cases diagnosed in Connecticut hospitals, 1935–53*

Years after diagnosis	Age at diagnosis					
	All ages	Under 45	45–54	55–64	65–74	75 and over
All stages combined						
0–1	83	88	86	82	79	72
1–2	69	74	71	69	66	57
2–3	54	65	62	59	56	47
3–4	52	57	55	52	47	37
4–5	46	52	50	46	41	32
5–6	41	49	46	41	37	22
6–7	37	46	42	37	30	19
7–8	34	43	39	34	26	17
8–9	31	41	37	31	23	13
9–10	29	39	35	28	21	10
10–11	27	37	33	26	18	8
11–12	25	35	31	25	16	6
12–13	24	34	29	24	15	5
13–14	22	33	27	22	11	4
14–15	20	30	26	19	10	2
Localized						
0–1	94	97	96	94	92	83
1–2	85	89	88	87	84	70
2–3	78	82	83	80	76	59
3–4	72	77	78	75	68	49
4–5	67	73	74	69	61	44
5–6	61	70	70	63	55	33
6–7	56	69	66	58	45	28
7–8	53	67	63	55	39	25
8–9	49	64	61	49	35	19
9–10	46	62	58	47	31	16
10–11	43	60	56	43	28	13
11–12	41	57	52	40	27	10
12–13	38	56	47	39	24	7
13–14	36	54	45	35	20	6
14–15	33	51	42	34	19	4
Regional						
0–1	83	86	87	83	81	70
1–2	65	68	67	64	63	53
2–3	52	55	54	51	50	44
3–4	42	46	46	41	38	31
4–5	37	39	41	35	33	25
5–6	32	36	36	31	29	16
6–7	28	32	32	27	24	15
7–8	25	30	29	23	21	13
8–9	23	28	27	22	18	9
9–10	21	25	25	20	16	6
10–11	19	24	24	18	13	4
11–12	18	22	23	18	11	4
12–13	17	20	21	18	11	4
13–14	15	20	20	16	5	2
14–15	13	18	19	13	4	0

TABLE 6.—*Follow-up year in which an annual survival ratio of 98 or better was reached; percentage of survivors at beginning of year: breast-cancer cases diagnosed in Connecticut hospitals, 1935–53*

Age at diagnosis	Follow-up year in which a ratio of 98 was reached*			Percentage of survivors at beginning of that year		
	All stages	Localized	Regional involvement	All stages	Localized	Regional involvement
All ages	12th†	8th	12th†	27	56	19
Under 45	14th†	7th	‡	34	70	‡
45–54	14th†	9th	15th	29	63	20
55–64	12th†	8th	11th†	26	58	20
65–74	10th	10th	‡	23	35	‡
75 and over	7th	7th	‡	22	33	‡

*Ratios may fluctuate around 98 beyond the selected interval. One could smooth the trend of annual survival ratios by mathematical manipulation. However, in this instance, the advantage to be gained through smoothing does not seem to warrant the computing involved. Inspection of the figures seems to be sufficient.

†Additional data are needed to evaluate the significance of the apparent reversal in the trend of ratios beyond this interval. *See* table 4.

‡ Not reached by the 15th year.

to evaluate the significance of the observed trend reversal and to determine the cause.

Discussion

It is clear from the foregoing discussion that, although the 5-year survival rate is a useful index for comparing different groups of patients, survival for 5 years does not necessarily mean that the excess mortality risk due to cancer has been eliminated for the remaining members of a patient cohort. The data indicate that mortality may continue to be "above normal" for a considerable period beyond the 5-year point.

An arithmetic index for evaluating the survival experience of cancer patients in terms of the survival experience of the general population has been presented. This index, the annual survival ratio, provides a basis for selecting an "acceptable" continuing survival rate. For illustrative purposes, it has been suggested that a ratio of 98 percent might be considered acceptable for breast cancer. Although 98 seems to be reasonably close to 100, an annual survival ratio must be evaluated in terms of its long-term effect. For example, an annual survival ratio of 98 percent results in a ratio of 90 percent over a 5-year period and a ratio of 82 percent over a 10-year period.[9] This means that over a period of 10 years an annual 2 percent deficit in the number of survivors will result in 18 percent fewer survivors, which is a sizable reduction. Furthermore, as was pointed out, an annual survival ratio of 98 percent will result in a 25 percent reduction

[9] $(0.98)^5=0.90$; $(0.98)^{10}=0.82$.

in life expectancy for the average breast-cancer patient. Many clinicians may deem this to be excessive and completely unacceptable.

In any event, the trend of annual survival ratios provides insight into the survival pattern for cancer patients. The data in table 4 indicate that in this series of breast-cancer patients the annual survival ratios for localized cases leveled off in the 8th year after diagnosis. Among the regional cases, however, the upward trend of annual-survival ratios reached a peak in the 13th year and then turned down. This differential trend for localized and regional cases may have significant implications for theories concerning the carcinogenic process. However, additional data are needed to confirm the reliability of these findings[10] and to analyze the relationship between the trend of annual survival ratios and the distribution of causes of death. At least two questions arise: 1) what cause or causes are responsible for the fact that the relative survival rate for localized breast cancer levels off below the general population level; and 2) what cause or causes are responsible for the reversal among regional cases in the trend of annual survival ratios?

Additional data are being tabulated to provide information on the long-term survival experience of patients with various forms of cancer. A study is also under way to determine the manner in which the distribution of causes of death among cancer patients changes with survival time and to determine the relationship of this distribution to the mortality rate. It is of interest to ascertain whether "above normal" mortality, 5 or more years after treatment, is due to cancer *per se*, to specific causes referable to the therapy employed, or to a generally increased susceptibility to the disease.

The volume of data required for the statistical analysis involved in this discussion generally cannot be obtained in a single hospital or clinic. The Connecticut program serves to illustrate a successful cooperative effort by a group of hospitals. However, the hospitals cooperating in a coordinated cancer registry program need not be located within a limited geographic area, as in Connecticut. The success of a cooperative program does not depend on geography but rather on completeness of registration and follow-up in each individual hospital or clinic, and on the maintenance of uniform information in accordance with a well-developed set of standardized definitions and procedures.

The large series of cases (9,121 breast-cancer patients) and the extensive follow-up information (15 years) made available by the Connecticut Cancer Record Register made possible the calculation of the annual survival ratios discussed. Similar analyses will be prepared for various forms of cancer. However, even the large volume of material collected in Connecticut (more than 100,000 cancer cases to date) will be insufficient for a truly comprehensive study of cancer end results. A very large body of material is needed to make possible the analyses of long-term survival results for cancers originating in various organs and tissues, with respect to such factors as histological type, stage of disease, method of therapy, clinical condition, etc.

[10] *See* table 4, footnote.

In order to make available the amount and the kind of information needed for a comprehensive study of therapeutic end results, the National Cancer Institute is sponsoring a cooperative program in which the resources of many cancer registries are being pooled.[11] Within the next two years, uniform information will be available on approximately 500,000 cancer patients. Each year, data on about 50,000 new cancer cases will be added and the information on previously reported cases will be brought up to date. This cooperative program will not only make possible a variety of detailed statistical analyses, but will also provide a basis for selecting special groups of cases for detailed case-history studies.

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[11] Six central registries, like the one in Connecticut, and 11 individual hospital registries are currently participating in this program, which is being administered through the Cancer Chemotherapy National Service Center.

Amyloidosis in Mice Exposed to Daily *Gamma* Irradiation [1, 2]

S. Lesher, D. Grahn, *and* A. Sallese, *Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois*

Previous reports have indicated that the liver and kidney are relatively resistant to acute radiation in the 30-day lethality range (*1–5*). Few studies have considered the late survivors of sublethal and fractionally lethal doses. Furth *et al.* (*6*) found a high incidence of nephrosclerosis in mice surviving up to 30 months after exposure to doses above 500 r from a nuclear detonation. Although nephrosclerosis was not found in their nonirradiated mice it is known that degenerative disease does occur in both the liver and kidneys of old mice.

During the course of experiments associated with the present investigation (*7*) it was observed that many mice receiving low daily doses of γ-irradiation (6 r/day or 12 r/day) show grossly detectable lesions in the liver and kidneys which, upon microscopic examination, were found to contain amyloid. Similar lesions also occur in the nonirradiated controls. Dunn (*8*) reports a high incidence of spontaneous amyloidosis and renal disease in strain A and hybrid ABC mice. Although numerous studies (*9*) have shown that amyloid may be produced experimentally in the mouse by a variety of chemical agents, there are no published accounts of amyloid infiltration following irradiation. In this report the histological nature of liver and kidney changes following amyloid infiltration is described and their relationship to irradiation is discussed.

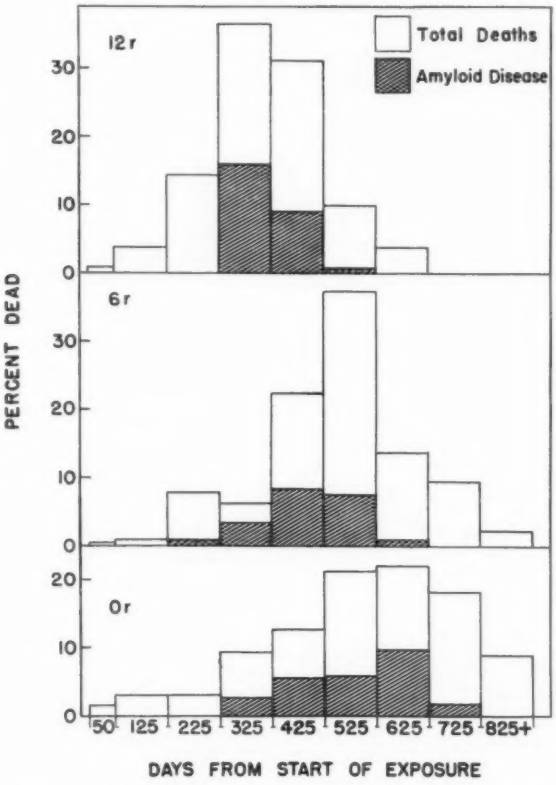
Materials and Methods

The study of incidence and type of lesions was based upon examination of 144 LAF_1 mice that had received 6 r per day of γ-irradiation, 132 that had received 12 r per day, and 223 nonirradiated controls.

Each group contained equal numbers of male and female LAF_1 mice. They were maintained 3 of a sex in a cage and allowed food and water *ad libitum*. All mice were 100 days of age at the start of exposure. Cages and *gamma* facilities have been previously described (*10*). The desired γ-ray doses were delivered by a 10 C Co^{60} source. These animals were exposed for the duration of life to the indicated doses of γ-irradiation

[1] Received for publication June 28, 1957.
[2] This work performed under the auspices of the U. S. Atomic Energy Commission.

and were autopsied at death (*7*). The liver and kidney condition was described grossly and representative tissue samples were fixed for histological study. In addition, a limited number of γ-irradiated mice were periodically sacrificed for the demonstration of amyloid in freshly fixed tissues. Two fixatives were used: (*a*) 20 : 2 : 1 mixture of 70 percent ethyl alcohol, 20 percent neutralized formalin, and glacial acetic acid; (*b*) neutral 10 percent formalin.

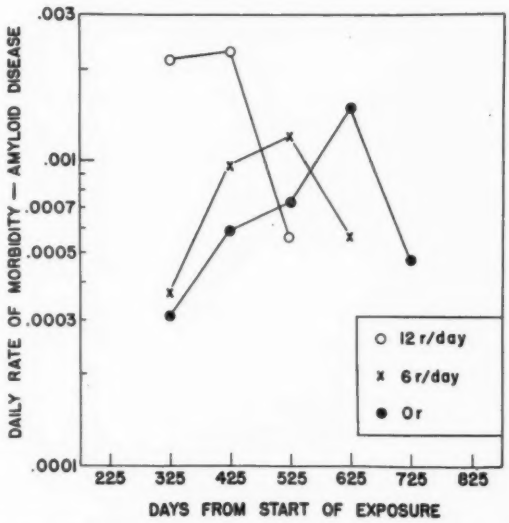


TEXT-FIGURE 1.—Frequency distribution of mice dying during 100-day intervals after the start of irradiation. All mice were 100 days of age at start of mortality experience.

All tissues were sectioned and stained routinely with hematoxylin and eosin. In addition, tissues from sacrificed animals were stained with toluidine blue, 1 : 2,000 aqueous solution buffered at *p*H 4.5 and 7.0, and by the periodic acid-Schiff (PAS) technique (*11*, *12*). For a more positive identification of amyloid, formalin-fixed frozen sections, cut by the Adamstone-Taylor cold-knife method (*13*, *14*), were stained with methyl violet and congo red.

Observations

The liver and kidneys of a high precentage of irradiated and nonirradiated mice dying within the mid-lifespan of each group contained amyloid (text-fig. 1). The peak incidence was higher and was reached earlier in the irradiated animals. This peak occurs in the 376- to 475-day period following the start of irradiation in mice receiving 12 r per day, and in the 476- to 575-day period in those receiving 6 r per day, whereas in the nonirradiated controls it is reached between 576 and 675 days (text-figs. 1 and 2). At 24 r per day the incidence of amyloid disease was negligible (3 cases among 210 mice), and at higher levels it apparently did not occur.



TEXT-FIGURE 2.—Rates of morbidity of amyloid disease calculated on the basis of 100-day interval lengths; morbidity rates on log scale. All mice were 100 days of age at start of exposure.

Although amyloid involvement was most pronounced in the liver and kidney it was not confined to these organs, and, in advanced stages, was found also in the spleen and adrenal glands.

Liver

Amyloid first accumulates in the peripheral sinusoids adjacent to the portal vein. In later stages all sinusoids frequently are involved. In the liver, amyloid stains a light pink with PAS and with methyl violet but takes on an orange-red appearance with congo red.

Massive accumulations such as those found in the kidney do not form in the liver. In late stages, amyloid is found between the sinusoidal endothelium and the liver cells, resulting in pressure atrophy of the latter.

The liver cells in amyloid-infiltrated areas contain little or no glycogen. Even in amyloid-free areas in the center of the lobule there is much less glycogen than is normally found in the non-amyloid livers (fig. 1).

Kidney

The progress of amyloid disease in the kidney and the sequence of changes produced by amyloid accumulation follow a well-defined pattern. Amyloid is first detected in the glomerular region (fig. 6). In this early phase, the kidney tubules and the blood vessels that are not in close proximity to the glomeruli do not contain sufficient amyloid to give a positive methyl-violet or congo-red reaction. The kidneys from such mice are normal in gross and microscopic appearance.

As amyloid accumulates in the glomerular capillaries, the capillary tuft completely fills the enveloping Bowman's capsule. From this stage on, there is a progressive increase in amyloid in the interstitial connective tissue of the cortex (fig. 2) and medulla (fig. 3) and an apparent infiltration into the adjacent tubules. Ultimately, glomerular circulation is completely blocked by amyloid deposits and atrophy of the associated tubules occurs. Dense-staining amyloid casts, which retard the movement of excretory fluids, form at all levels of the kidney tubules (fig. 3). The excessive accumulation of fluids distends the glomerular capsules and cortical tubules producing numerous cysts that frequently protrude from the kidney surface (figs. 7 and 8).

In advanced amyloid disease hypertrophy of the connective-tissue stroma frequently accompanies the atrophy of the kidney nephron. In the terminal stage there appears to be a change in the chemical nature of the amyloid deposits since, although they still give an intense PAS-positive reaction, they stain either faintly or not at all with methyl violet or congo red. However, more reactive amyloid is usually found in many of the remaining glomeruli and associated blood vessels.

Spleen

Amyloid in the spleen stains an intense orange-red color with congo red. In severe involvements dense homogeneous amyloid masses completely obliterate the structural organization of the red pulp (fig. 4). In such spleens the germinal centers are noticeably reduced. In the terminal stages, there is an over-all structural atrophy of the spleen; however, this does not necessarily result in a reduction of spleen size, since the accumulation of amyloid frequently is sufficient to replace the normal cellular and intercellular material.

Adrenal Glands

In the adrenal glands, amyloid stains a light pink with congo red and by the PAS technique. It is deposited in an area between the cortex and the medulla. With the increase in amyloid, the adjacent cells of the cortex and medulla atrophy.

In addition to the observations made on advanced stages of amyloid

disease in which amyloid deposits were identified in the tissues at death, a number of cases were found in another experimental series of LAF_1 mice given 12 r per day and sacrificed at 30-day intervals from the start of exposure at 100 days of age (*15*). The earliest cases of amyloid disease were found in the mice sacrificed at 270 and 300 days. The liver and kidneys of several mice at each of the time levels contained amyloid. Although the over-all degree of amyloid involvement was low, it was relatively high in the liver, where it was localized in the peripheral region of the lobules (fig. 5). Amyloid deposits in the kidney, on the other hand, were restricted to the glomerular capillaries (fig. 6).

Discussion

Despite extensive recent studies, little has been added to our understanding of the etiology and pathogenesis of amyloid disease. In addition to the association of amyloidosis with degenerative diseases and chronic inflammation, amyloid deposits are frequently found in tumor-bearing organs (*16*). Multiple myeloma is often associated with atypical amyloid deposits (*17–19*), and Dunn (*8*) found that the histological structure of mouse pulmonary tumors is often obscured by massive amyloid infiltration. In a recent study of radiation-produced gastric neoplasms, amyloid was identified in the walls of blood vessels lying in the submucosal connective tissue within the tumor area (*20*). The relationship between amyloid synthesis and tumor growth is unkown; however, it is possible that the role of a developing tumor in the production of amyloid is similar to that of other chronic diseases.

Clinically, amyloid disease is classified into two principal types; (*a*) secondary amyloidosis which is associated with chronic suppurative diseases; (*b*) primary amyloidosis in which amyloid deposits are widely distributed throughout a variety of tissues but there is no definite knowledge as to the etiological agent. In the present study amyloidosis was not associated with either chronic suppurative disease or tumor growths, therefore it is concluded that the amyloid infiltration observed in these mice is a primary amyloidosis.

Amyloidosis can be experimentally produced in animals by a wide variety of treatments. Kuczynski (*21*) first produced amyloid disease in mice through repeated injections of sodium caseinate. Other workers have produced amyloidosis by injecting or feeding such substances as nucleic aicd (*22*), cholestrol (*23*), nutrose (*24*), alloxan (*25*), and nitrogen mustard (*26*). The extreme chemical diversity of these and other substances capable of producing amyloid suggests a nonspecific reaction; and it is unlikely that all these substances stimulate or inhibit the same metabolic system. Hence, it is not surprising that exposure to radiation leads to the production of amyloid, particularly when exposure is sufficiently protracted to permit animals to live until they reach a susceptible age, but at the same time high enough to affect physiological reactions. Regardless of the etiological agent, amyloid infiltration, once initiated, is apparently an irreversible process.

The incidence of amyloid disease in irradiated and nonirradiated animals, as summarized in text-figures 1 and 2, indicates a definite dose-dependence. The increasingly shorter lifespan of mice receiving 24 r per day, and above, explains the low incidence or complete absence of amyloid disease. Irradiation apparently accelerates the course of the disease but does not appreciably shorten the induction or so-called latent period, since amyloid first appears at approximately 275 days in all 3 experimental groups. It is of particular interest that amyloid disease in mice is predominantly a disease of the mid-lifespan. During this period, the percentage of animals that died with amyloid disease was quite high; *e.g.*, in the 12 r per day series, approximately 50 percent of the mice dying between 275 to 375 days after the start of irradiation had amyloidosis. Late in life, regardless of radiation history, the incidence of the disease is very low and it possibly does not occur among the last 10 percent of the deaths.

In this investigation observations were restricted to the liver, kidney, spleen, and adrenal; however, amyloid deposits accumulate throughout the animal in late stages of the disease giving some support to the infiltrative hypothesis. Other investigators (*6*, *9*, *27*) have found amyloid deposits in the dermis of the skin, eye, heart, lungs, nerve ganglia, and tongue.

In certain strains of mice there is a definite genetic predisposition for primary amyloidosis (*28*). Heston found that the incidence was approximately 100 percent in strain A and 18.2 percent in strain L, whereas in the LAF_1 it was 27.5 percent. The incidences in the 3 LAF_1 series (6 r/day, 12 r/day, and nonirradiated controls) used in this investigation were in close agreement with Heston's data; hence, it would appear that the genetic susceptibility to amyloid disease is a dominant factor in establishing the incidence regardless of the evocator. Irradiation increases the morbidity rate in the early periods without increasing the total incidence.

Although mucoproteins are normal constituents of all connective tissue, amyloid, which is also a protein-polysaccharide complex, is considered pathologic. The origin of the two moieties and the mechanism by which they are coupled is unknown. The coupling is believed to result from an immunological-type reaction (*29*), but the nature of the antigen or antigens involved has not been conclusively established. Until recently it was thought that only the protein molecule was plasma borne; however, recent studies suggest a relationship between a circulating abnormal protein-polysaccharide and amyloid (*30*). Since amyloid deposits frequently accumulate in the interstitial connective tissue of the infiltrated organs, and since the connective-tissue fibrocytes are probably involved in the synthesis of mucoproteins, the hypothesis implicating the connective tissue in amyloid formation (*31–34*) is not unreasonable and has met little opposition. This theory does not completely account for the high concentration of amyloid in organs containing very little or no connective tissue; *e.g.*, the kidney or the central nervous system.

The study of early stages of the amyloidosis found in the series of irradiated mice (12 r/day) sacrificed at 30-day intervals shows that amyloid first appears in the liver and reaches a high degree of involvement before it can be identified in the kidney. As the disease progresses, dense masses of amyloid accumulate in the kidney and spleen. The accumulation of amyloid in the medullary tubules of the kidney blocks the movement of excretory fluids and terminates in dysfunction and death. Although amyloid is probably synthesized in numerous organs, including the kidney, it is reasonable to assume that it may be carried from the more productive organs to other sites by the blood plasma. The finding of a circulating protein-polysaccharide lends support to this suggestion (*30*, *35*). Otherwise it is difficult to account for the high concentration of amyloid found in the kidney in advanced cases of amyloidosis or to explain its presence in the brain.

Summary

Amyloid disease is a common lesion observed both in control and in LAF_1 mice exposed to low daily doses of γ-irradiation. Amyloidosis first appears about 9 months after the start of exposure to 6 and 12 r per day of γ-irradiation, which continues for the duration of life. The initial appearance in control mice is at a comparable time but the peak incidence occurs earlier in the exposed groups. Late in life the morbidity rate decreases sharply in all populations. The infiltrative nature of the disease produces the most severe degenerative sequelae in the kidney, although, in advanced cases, extensive atrophy of the hepatic cells and splenic follicles is observed.

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PLATE 91

The tissues represented by figures 1 through 4, inclusive, are from a mouse that had received 12 r per day of γ-rays for 363 days (total dose 4,356 r). Irradiation was delivered by a 10 C Co^{60} source. Amyloid disease in this animal was in an advanced but not a terminal stage. It should be emphasized that in a similar advanced stage of amyloid infiltration the pattern of involvement and the concomitant organ damage is essentially the same both in irradiated and nonirradiated animals.

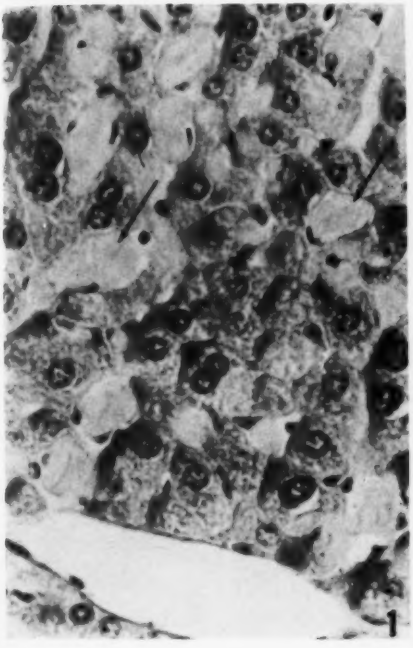
FIGURE 1.—Liver section showing amyloid deposits. Atrophy of the hepatic cells well advanced. *Arrows* point to amyloid material which is distributed throughout the lobule. Stained by the periodic acid-Schiff reaction. × 435

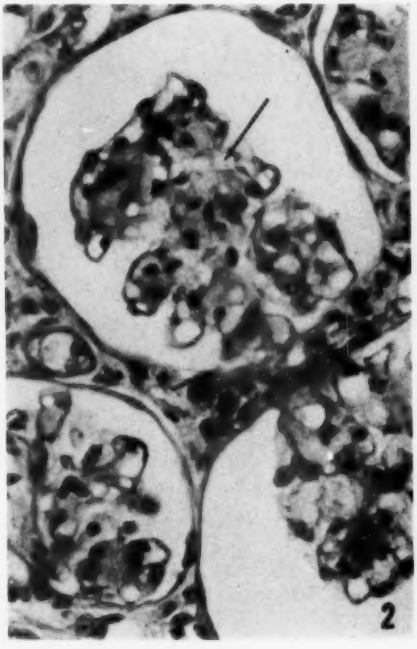
FIGURE 2.—Cortex of the kidney stained with amyloid-specific congo red. Amyloid is present in the glomerular capillaries and interstitial connective tissue. Bowman's capsules surrounding the glomerular capillaries have been distended by excretory fluids forming numerous small cysts (fig. 7). × 435

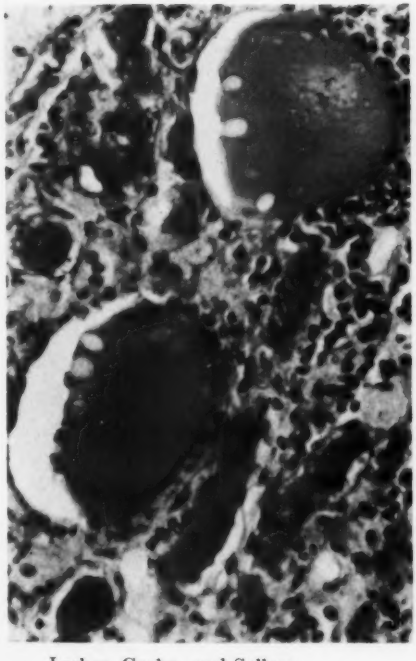
FIGURE 3.—Outer medullary region of PAS-stained kidney of which the cortex is shown in figure 2. Amyloid deposits are found in the medullary capillaries, kidney tubules, and in the interstitial connective tissue. Amyloid concentration in advanced stages of amyloidosis is always extremely high in the medulla. Numerous dense-staining casts are characteristic of this stage of the disease. × 435

FIGURE 4.—Spleen stained by PAS reaction showing that amyloid is confined for the most part to the red pulp. Sinusoids are blocked and structure obliterated. × 435

Photomicrographs by Atlee Tracy.







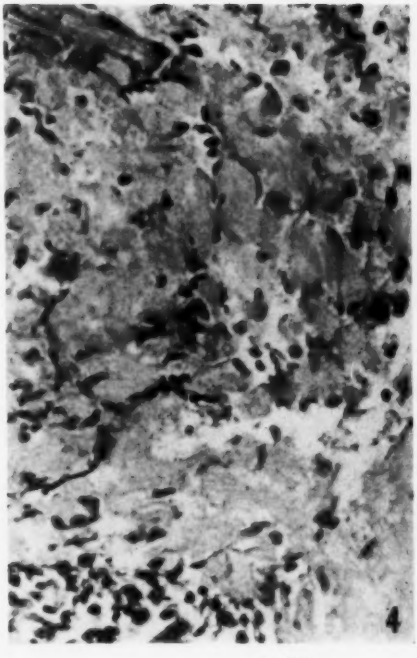


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PLATE 92

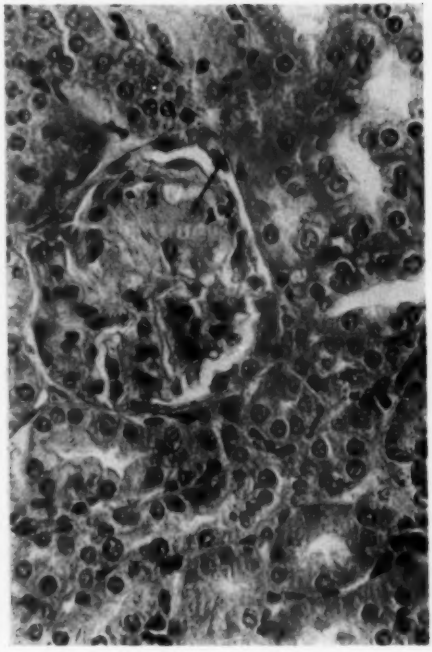
FIGURE 5.—This section is from the liver of a mouse sacrificed after exposure to 12 r per day for 270 days with a total accumulated dose of 3,240 r of γ-irradiation. *Arrows* point to amyloid deposits between the liver cords. Hepatic cells in this area have already begun to atrophy.

FIGURE 6.—The kidney from above mouse (fig. 5) contained very little amyloid. Amyloid is confined to the glomeruli where it is present in low concentration. Amyloid could not be detected in other kidney structures. Stained by the periodic acid-Schiff reaction. × 435

FIGURE 7.—Low-power sagittal section of kidney from which figures 2 and 3 were taken. Small cysts can be detected in the corticular region. × 5

FIGURE 8.—Low-power sagittal section of kidney from nonirradiated control mouse sacrificed at 610 days of age. Amyloid disease was in a terminal stage. In the kidney almost all glomeruli were completely atrophied and prominent cysts were grossly visible. The infiltration of the liver was equally severe and the damage to hepatic cells extreme. × 5

Photomicrographs by Atlee Tracy.



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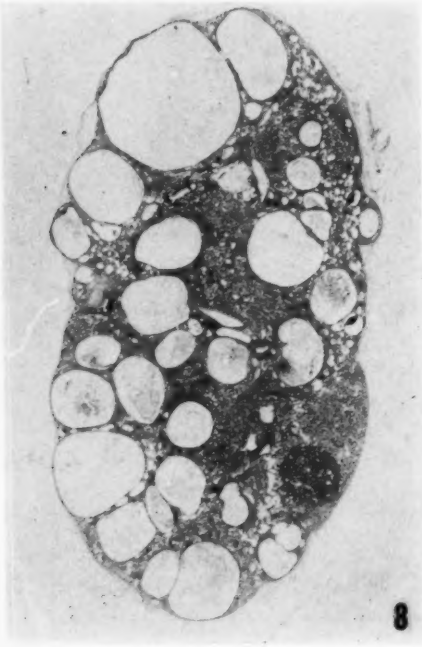

8

Comparison of the Relative Effectiveness of Folic Acid Congeners Against Advanced Leukemia in Mice [1]

ABRAHAM GOLDIN, JOHN M. VENDITTI, STEWART R. HUMPHREYS, *and* NATHAN MANTEL, *Laboratory of Chemical Pharmacology, and Biometry Branch, National Cancer Institute,*[2] *Bethesda, Maryland*

An assay procedure has been described previously for comparing the effectiveness of alternative therapies in increasing the survival time of mice with advanced leukemia (L1210) (*1*). Hybrid male mice (BALB/cAn × DBA/2J) are inoculated in the right hind leg with a standard dose of leukemic cells, and treatment is initiated when the local tumor, at the site of inoculation, is approximately 8 to 12 mm. in diameter. At this time the disease is systemic, and untreated control mice succumb in 1 to 4 days. The assay procedure requires testing at dose levels ranging from well-tolerated doses to those producing toxicity.

Employing this assay, the folic acid antagonist, amethopterin, was found to be more effective than various other antileukemic agents currently employed in studies of animal and clinical leukemia (*2*).

A program was therefore instituted to evaluate the relative effectiveness of a series of folic acid analogues.[3] One of these, 3′,5′-dichloroaminopterin was more effective than aminopterin and almost as effective as amethopterin (table 1), suggesting that the corresponding dichlorinated derivative of amethopterin be investigated.

The amethopterin derivative, 3′,5′-dichloroamethopterin has marked activity against advanced leukemia (L1210) in mice (table 1; text-fig. 1). Text-figure 1 summarizes the relationship between median survival time and dosage in a comparison of amethopterin and 3′,5′-dichloroamethopterin on 4 different schedules of treatment. In this experiment, treatment was initiated on the 8th day following leukemic inoculation and continued through the 37th day. The untreated controls showed a median

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

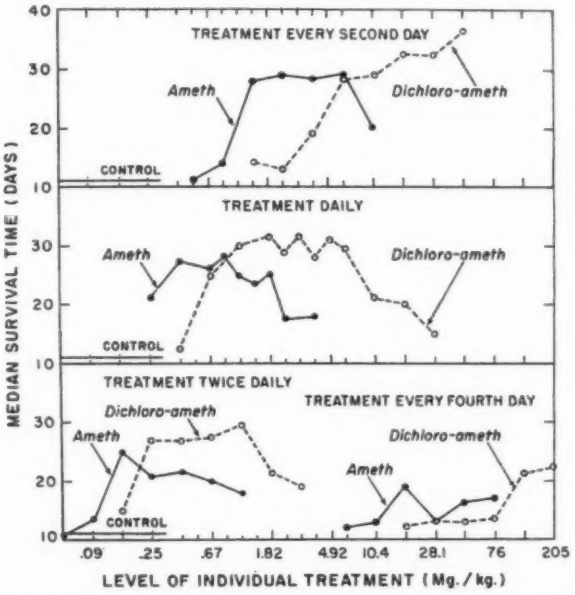
[3] The various folic acid (pteroylglutamic acid, PGA) congeners were kindly provided by the Research Division, American Cyanamid Company, Pearl River, New York: amethopterin (methotrexate, Lederle 7-5054 and Calco R-2629-73); aminopterin (Calco R-2727-199); 3′,5′-dichloroaminopterin (American Cyanamid R-2795-145, Cl 14, 379); 3′,5′-dichloroamethopterin (American Cyanamid 3075C); N^{10}-ethylaminopterin (American Cyanamid 3161C); aminoteropterin (American Cyanamid 3004L); adenopterin (American Cyanamid 3007C). Following the completion of these experiments it was learned from Dr. Robert B. Angier of the Research Division of American Cyanamid Company that the 3′,5′-dichloroamethopterin contained the corresponding monochloroamethopterin as an impurity. Pure samples of monochloroamethopterin and dichloroamethopterin have now been synthesized by the Research Division, American Cyanamid Company and are in the process of being tested. For the synthesis of the samples of the dichlorinated derivatives employed in the current studies see (*3*).

TABLE 1.—*The relative effectiveness of folic acid congeners against advanced leukemia L1210 (daily treatment)*

Experiment	Initial day* of treatment	Compound	Optimal daily dose (*mg./kg.*)	Median survival time (*days*)
1	8	Amethopterin	1. 21	24. 0
		3′,5′-Dichloroaminopterin†	3. 35†	21. 5†
		Aminopterin	0. 16	16. 5
		Controls		11. 0
2	9	Adenopterin	0. 75	26. 5
		Amethopterin	0. 75	26. 0
		3′,5′-Dichloroaminopterin	1. 25	24. 0
		Aminoteropterin†	0. 45†	17. 0†
		Controls		11. 0
3	12	3′,5′-Dichloroamethopterin	3. 50	31. 0
		N^{10}-Ethylaminopterin	1. 25	29. 0
		Amethopterin	0. 75	27. 5
		Controls		15. 0

*Day 0=day of leukemic inoculation.

†The optimal dose was not attained. Figures shown represent results with the lowest dose level actually tested. Each compound was employed in a wide range of doses. Comparisons of effectiveness are made at the dose level of each drug which elicited the maximum extension in the median survival time. Ten mice per group.



TEXT-FIGURE 1.—Comparison of the effectiveness of amethopterin and 3′,5′-dichloroamethopterin against advanced leukemia (L1210) on various schedules of treatment. Each point represents the median survival time in a group of 10 mice. Treatment was initiated 8 days following leukemic inoculation. The concentration of the leukemic inoculum employed was 1.14×10^6 cells per mouse and was 1,000 times the inoculum which resulted in 100 percent lethality from tumor growth in untreated mice.

survival time of 11 days and the last mouse died on the 12th day. On each of the 4 schedules of treatment 3′,5′-dichloroamethopterin, was more effective than amethopterin in increasing the life span of the leukemic mice. For each schedule, the increased effectiveness was evident over a wide dose range. With 3′,5′-dichloroamethopterin, unlike amethopterin, regression of the local tumor at the site of leukemic inoculation was observed at dose levels which did not cause concurrent loss in body weight.

The most extensive median survival time (36.5 days) was achieved with 3′,5′-dichloroamethopterin administered every 2 days at a level of 46 mg. per kg. This represents an approximate ninefold increase in the remaining lifetime from the initiation of treatment. At the time the treatment was discontinued, a total of 16 mice that had received 3′,5′-dichloroamethopterin were still alive, while there were only 2 remaining mice in the amethopterin-treated groups. Three of the 3′,5′-dichloroamethopterin-treated mice survived beyond 50 days.

Two other folic acid congeners, adenopterin and N^{10}-ethylaminopterin, showed antileukemic effectiveness comparable with that of amethopterin (table 1). Aminoteropterin was approximately as effective as aminopterin. Although amethopterin and aminopterin are the 2 folic acid derivatives commonly employed clinically, the current data suggest that additional derivatives be synthesized and studied in experimental leukemia, for potential clinical application.

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(*3*) COSULICH, D. B., SEEGER, D. R., FAHRENBACH, M. J., ROTH, B., MOWAT, J. H., SMITH, J. M., JR., and HULTQUIST, M. E.: Analogs of pteroylglutamic acid. VI. 3′,5′-Dihalopteroyl derivatives. J. Am. Chem. Soc. 73: 2554–2557, 1951.

Minimum Standards for the Commercial Production of Randombred and Inbred Laboratory Mice (Revised September 22, 1957) [1, 2, 3]

Purpose and Aim of the Mouse Producers Accreditation Program

The laboratory mouse is the tool of choice in a vast number and variety of studies aimed at the prevention and cure of human diseases. It is also widely utilized in the testing of innumerable drugs and biological products for their usefulness in alleviating human suffering.

In medical research it is of great importance that the tools used be as uniform and standardized as possible, in order that the results obtained in one laboratory may be repeatable in a different laboratory. Uniformity of the tools used in research also increases the accuracy of the results obtained. In addition, it speeds research by decreasing the number of repetitive experiments needed to ascertain that a particular result is not merely due to chance.

Two types of uniformity must be considered in the case of the laboratory mouse: genetic uniformity and environmental uniformity.

Genetic uniformity may be considered from two different aspects depending on the mating system employed. In the case of the genetically undefined white mouse, commonly produced commercially, consistent variability can be achieved by maintaining all commercial colonies under a system of rigidly randomized matings. This will insure that all lots of white mice obtainable commercially will be uniformly variable in their genetic constitution. Experiments utilizing these white mice could then be designed on the basis of their uniform genetic variability, eliminating the present source of error due to uncertainty of the degree of genetic variability of different lots of mice. It cannot be too strongly emphasized that the uniform genetic *variability* of randombred white mice is just as important as the uniform genetic *invariability* of inbred strains of mice.

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[1] Prepared by the Committee on Standards of the Institute of Laboratory Animal Resources, National Academy of Sciences, 2101 Constitution Ave., N. W., Washington 25, D. C., for the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Bethesda 14, Maryland.

[2] Members of the Committee are: T. C. Byerly, *Chairman;* T. Aarons; R. H. Barnes; N. R. Brewer; L. R. Christensen; C. N. W. Cumming; R. J. Flynn; H. L. Foster; G. E. Jay, Jr.; J. E. Williams; J. W. Wilson.

[3] Copies of the "Minimum Standards" may be obtained from the Institute of Laboratory Animal Resources or from the Cancer Chemotherapy National Service Center.

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The genetic constancy of inbred strains of mice can only be maintained by continuing brother × sister matings, beyond the twentieth generation of such matings, for the entire life of the strain. All animals within a strain should be as closely related as possible, tracing back to only a few ancestors in every generation. The usefulness of different inbred mouse strains arises out of the specifically defined physiological characteristics of each strain. Thus, for example, a specific transplantable tumor will grow only in mice belonging to a specific inbred strain and not in any other strain. Mice of different inbred strains may have different susceptibilities to certain infectious diseases or to cancer formation. The probability that all mice of a specific inbred strain will show the same response to a specific experimental treatment is very high, certainly far higher than that for randombred mice under similar treatment. Thus, by utilizing the very low degree of genetic variability of inbred mice, experiments can be designed in which almost all variability in the results can be attributed to the experimental treatment and other environmental conditions.

Environmental uniformity, equally as important as genetic uniformity, is in practice more difficult to attain. Since laboratory mice are produced in different localities by different breeders and under different conditions, it is essentially impossible to produce all mice under environmental uniformity. However, it is both possible and practical to approach this ideal condition. This can be accomplished, for both randombred and inbred mice, by maintaining the animal colony under optimal conditions of cleanliness, nutrition, temperature and humidity, and prevention of disease. Although definitive experimental studies on the best cage design, nutrition, and other problems of colony managment are urgently needed, a beginning towards the establishment of optimal environmental conditions in all commercial laboratory mouse colonies can be made by utilizing the past experience of academic and commercial mouse producers. It is out of this experience that the minimum standards presented herewith have been evolved. It should be noted that these are *minimum* standards. It is assumed that all producers will want to establish practices in their colonies which will enable them to raise mice under optimal conditions which will considerably surpass those outlined in the standards.

As both the producers and the accrediting agency gain experience in the administration of the program of accreditation of mouse producers, changes in the minimum standards will have to be made. As the standards are presented now, they are not intended to prevent any producer from trying out or adopting practices not specifically mentioned in the standards if these practices are designed to improve the conditions under which the mice are raised. In fact it would be very helpful to the accreditation program if producers would acquaint the accrediting agency with animal husbandry practices which they find to be superior to those mentioned in the standards.

The sole aim of these standards and of this accreditation program is to

aid the commercial mouse breeder in the production of more uniformly healthy, well-fed mice of known genetic background and variability. Such mice will aid immeasurably in increasing the reliability of experimental results obtained in medical research and will thus be of great benefit to mankind.

To the User of Laboratory Mice

The mice produced by accredited breeders under the conditions required by the standards of accreditation are of superior quality. They will give more dependably uniform experimental results and more uniform bioassay results than mice produced under substandard conditions. This superior quality can only be maintained by employing breeding and husbandry practices, which are more expensive than those previously used. It is therefore impossible for an accredited mouse producer to compete *on a price per mouse* basis with dealers and breeders producing mice of average or inferior quality. Since the mice used in any experiment are a minor cost item, it will behoove every investigator to specify to his purchasing agent that mice are to be obtained only from accredited producers.

To the Producer of Laboratory Mice

All methods of housekeeping, nutrition, disease diagnosis and prevention, genetics, and record-keeping—including those recommended in these standards—used in the commercial production of randombred and inbred laboratory mice must be approved by the accrediting agency. The accrediting agency may give provisional approval to producers who give assurance of conformity within a definite and reasonable period of time.

Producers who meet these standards will receive an official statement from the accrediting agency which they may use as evidence that their products conform to good production standards. Added cost of adherence to these standards properly may be reflected in price.

Facilities and Sanitation

Facilities, equipment, and sanitary practices shall be designed to provide minimal opportunity for transmission of diseases and parasites from the population in one cage to populations in other cages, and to subsequent populations which may occupy the same cage. Such practices will minimize the opportunity for the transmission of diseases and parasites into the producers' premises and from the producers' premises to the users' laboratories.

Sanitization: To make physically clean and to remove and/or destroy agencies injurious to the health of laboratory animals.

Sterilization: The act or process of killing all living cells, especially microorganisms.

Caging and equipment:

1) Cages and racks shall be made of a smooth, corrosion-resistant, impervious, easily cleaned and sanitized material; *e.g.*, stainless steel,

plastic, or glass. If present cages or racks do not conform to these specifications, they shall be replaced by cages and racks of approved material within a period of time to be determined by the accrediting agency in consultation with the individual producer.

2) Minimum cage area, per nursing female, shall be 30 square inches.

Minimum cage area, per mouse up to 60 days old, shall be 8 square inches.

Minimum cage area, per mouse over 60 days old, shall be 10 square inches.

Maximum number of mice in any cage of any size shall be 30.

3) Water bottles or other devices, equivalent in protection of water against contamination and approved by the accrediting agency, shall be provided.

4) Feed hoppers constructed of corrosion-resistant material shall be suspended in each cage. All hoppers should be covered.

5) Animal room floors, walls, and ceilings should be made of a smooth, hard, impervious material that can be cleaned easily. New buildings should be constructed of materials other than wood.

Bedding:

1) Bedding shall consist of sawdust, shavings, or other material not readily eaten. Hay, straw, or shredded sugar cane shall not be used.

2) Bedding shall be protected from exposure to vermin prior to use. It should not be obtained from any unprotected storage source.

3) Bedding shall be dry when received.

4) Storage at the mouse breeder's establishment shall be under clean, dry, vermin-free conditions.

5) Bedding shall be changed weekly or oftener.

Buildings:

1) Animal room temperature should be maintained between 70° F. and 80° F. In the summer, buildings can be cooled by painting the roofs with aluminum paint, attaching sprinkler hose to roof, and using ventillating fans inside the building. Air conditioning is recommended.

2) Adequate, draft-free ventilation shall be provided.

3) Buildings shall be rodent-proof and vermin-free.

Cleaning:

1) Animal house floors shall be scrubbed with hot water and detergent once each week. Walls and ceilings shall be cleaned frequently, preferably by scrubbing with hot water and detergent.

2) Corridors and animal room floors shall be swept daily with a sweeping compound. Frequent dusting is recommended.

3) Cages, feed hoppers, water bottles and watering tubes, or other watering devices shall be very thoroughly cleaned with hot water and detergent or otherwise sanitized before new animals are placed in the cages.

a) All cleaning and sanitization methods used shall be examined for adequacy by the accrediting agency.

b) Mechanical cage washing machines (operating at 180° F.) used with an effective detergent are recommended. If these are used, no separate sanitization of cages is necessary under normal conditions.

c) It is recommended that all cages and watering devices be sanitized at least once each week.

4) The water bottle or other watering device removed from any cage shall be returned to the *same* cage unless it is sanitized in a manner approved by the accrediting agency. This is very important for the control of diseases.

5) All refuse and dead animals shall be placed in closed containers and removed from the premises daily. Incineration is recommended.

6) Refuse containers and covers shall be sanitized thoroughly after use and before being brought into the animal room.

Disease Diagnosis and Prevention

The introduction of new stock to any breeding colony must always be considered a hazard, and every breeder should use every precaution to protect his own stock. Each inbred nucleus colony will practice the strictest sanitary and disease-prevention measures and every effort will be made to deliver the breeding stock in an uncontaminated and disease-free condition.

1) All breeding stock obtained from any source, including the inbred nucleus, shall be quarantined upon receipt for at least 3 weeks in a room or building isolated from the rest of the mouse colony. It is recommended that during this period samples of these animals be tested for salmonellosis.

2) All persons entering the colony shall wash themselves very thoroughly, and dress in special shoes or clean shoe covers, and clean clothing or uniforms before beginning work in the animal rooms. The clothing, uniforms, shoes and shoe covers are to be worn only in the animal rooms and are not to be removed from the animal room until they are removed for laundering. The clothing, uniforms, and shoe covers shall be washed at least once a week.

3) Personnel handling animals should wash their hands thoroughly at frequent intervals. Hands should be washed immediately after handling sick animals.

4) Visitors should not be allowed in any animal room.

5) To prevent disease dissemination all mice shall be shipped and delivered in new and nonreturnable containers which have been approved by the accrediting agency. These containers shall be stored, prior to use, in clean quarters separate from the animal rooms.

6) Periodic examinations of samples of mice, taken from different cages in the colony, shall be made by competent diagnostic laboratories. A minimum of two examinations a year shall be performed at least 4 months apart. These examinations may include the following diseases for which

reports shall be submitted to the accrediting agency and to the producer by the diagnostic laboratory. Other examinations may be made at the request of the producer or upon request of users. The diagnostic laboratory shall be approved by the accrediting agency.

a) Salmonellosis
b) Ectromelia
c) Other pathological conditions found
   (1) No evidence of _____ since _____
   (2) Incidence of _____: _____% during preceding 6 months or 1 year.

7) Vaccination against ectromelia of all breeding and future breeding animals is recommended. If this procedure is followed, the requirement for periodic examinations for ectromelia of samples of mice from the colony may be waived by the accrediting agency.

8) Standard procedures for submitting materials for diagnosis, for the handling of sick animals, and for the control of epidemics shall be issued by the diagnostic laboratories and approved by the accrediting agency.

Genetics and Recordkeeping [1]

Mice distributed by accredited producers shall be identified with respect to ancestry and method of mating used in their production. Such mice may be certified by the accrediting agency.

Except in instances in which specific genetic or health requirements necessitate the introduction of new stock, maintenance of a closed population with no introduction of outside stock by each producer generally improves the uniformity of the stock and facilitates the maintenance of health in the colony.

The keeping of production records for each mating or breeding pen is recommended. Each cage card should include the mortality record (number of animals and dates of death).

Randombred mice are the progeny of matings, within the closed colony, of females and males generally from unrelated litters and different cages. A numerical randomization system based on cage and litter numbers approved by the accrediting agency should be used.

Inbred strains of mice are the progeny of mice resulting from brother × sister matings or from younger parent × offspring matings for twenty or more generations of such matings.

1) A small number of inbred nuclei established at different geographic locations and under the constant supervision of professional mammalian geneticists will supply all breeding stock to accredited commercial producers of inbred mice. There must be close liaison between the supervisor of the specific inbred nucleus and the associated producers supplied by this nucleus.

[1] *Basic reference:* Genetic Standards Chapter of Handbook of Laboratory Animals, Second Edition, Institute of Laboratory Animal Resources, National Research Council. In preparation.
Standardized Nomenclature for Inbred Strains of Mice, Cancer Res. 12: 602-613, 1952.

a) The inbred nucleus shall be propagated only by brother × sister matings or younger parent × offspring matings.
b) Complete records shall be kept for each animal in the inbred nucleus. A standard record card will be recommended by the accrediting agency.
c) To assure the retention of specific genetic and physiological traits by each inbred strain, adequate performance tests shall be conducted at frequent intervals in each inbred nucleus. Performance testing at the final production level will be done if necessary.

2) Production matings.
a) Foundation stock shall be obtained from one of the producers possessing an inbred nucleus.
b) Foundation stock shall be propagated by brother × sister or younger parent × offspring matings.
c) Only one generation of random matings within an inbred strain may be used for production of inbred mice for sale or for production of F_1 hybrids.

F_1 hybrid mice are the progeny from matings of male mice from one inbred strain and female mice from a different inbred strain. Specific F_1 hybrid combinations are useful for different specific laboratory purposes and are not generally interchangeable.

Nutrition [2]

1) Fresh feed shall be used. It should not be stored longer than 6 weeks after pelleting before use.

2) Feed shall be stored under clean, dry conditions in covered containers.

3) No feed containing unrendered meat scraps (a source of salmonellosis infections) shall be used. The feed manufacturer shall accept and use only rendered meat scraps which have been placed into clean—preferably sterile—containers (*e.g.*, five-ply kraft paper bags) immediately after rendering by the meat packer.

4) Each producer should make sure—especially if he obtains his feed from a local mill—that the feed he uses is:
a) within normal limits of naturally occurring hormone activity and free of diethylstilbestrol contamination;
b) free of additives containing medication or antibiotics;
c) checked for Salmonella.

The results of these assays should be clearly declared by the manufacturer.

5) If an investigator needs mice raised on a particular antibiotic-containing or other special diet, special arrangements shall be made with the mouse producer. This feed, and the mice raised on it, shall be kept in isolation from the regular feed.

[2] *Basic reference:* Publication of mouse nutrient requirements by the Subcommittee on Laboratory Animals, Committee on Nutrition, Agricultural Board, National Research Council. In preparation.

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